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Examiner D. Romeo

Filed: April 15, 1998

For: MODIFIED TNF- α MOLECULES, DNA ENCODING SUCH AND VACCINES
COMPRISING SUCH MODIFIED TNF- α AND DNA

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CLAIM FOR CONVENTION PRIORITY

Asst. Commissioner of Patents
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Sir:

The benefit of the filing date of the following prior foreign application filed in the following foreign country is hereby requested and the right of priority provided in 35 U.S.C. §119 is hereby claimed:

Denmark Appln. No. 0418/97 filed April 15, 1997

In support of this claim, filed herewith is a certified copy of said foreign application.

Respectfully submitted,

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Patent application No.: 0418/97

Date of filing: 15 April 1997

Applicant: Farmaceutisk Laboratorium Ferring A/S
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This is to certify the correctness of the following information:

The attached photocopy is a true copy of the following document:

- The specification, claims, abstract and drawings as filed with the application on the filing date indicated above.



Patent- og
Varemærkestyrelsen
Erhvervsministeriet

Taastrup 17 March 2000

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15. APR 1997

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Modificerede TNF α molekyler, DNA kodende for disse, samt vacciner indeholdende nævnte modificerede TNF α og DNA

Field of the invention

The present invention relates to human cytokine Tumor Necrosis Factor α (TNF α) molecules which has been modified so that they are capable of raising neutralizing antibodies towards unmodified human TNF α following administration of the modified TNF α molecule to the human host. The invention also relates to human TNF α vaccines based on said modified TNF α molecules. Further aspects of the invention will appear from the discussion below.

10 Background of the invention

Physiologically, the vertebrate immune system serves as a defense mechanism against invasion of the body by infectious objects such as microorganisms. Foreign proteins are effectively removed via the reticuloendothelial system by highly specific circulating antibodies, and viruses and bacteria are attacked by a complex battery of cellular and humoral mechanisms including antibodies, cytotoxic T lymphocytes (CTL), natural killer cells (NK), complement, etc. The leader of this battle is the T helper (T_H) lymphocyte which, in collaboration with the Antigen Presenting Cells (APC), regulate the immune defense via a complex network of cytokines.

T_H lymphocytes recognize protein antigens presented on the surface of the APC. They do not recognize, however, native antigen per se. Instead, they appear to recognize a complex ligand consisting of two components, a "processed" (fragmented) protein antigen (the so-called T cell epitope) and a Major Histocompatibility Complex class II molecule (O. Werdelin et al., Imm. Rev. 106, 181 (1988)). This recognition eventually enables the T_H lymphocyte specifically to help B lymphocytes to produce specific antibodies towards the intact protein antigen (Werdelin et al., supra). A given T cell only recognizes

a certain antigen-MHC combination and will not recognize the same or another antigen presented by a gene product of another MHC allele. This phenomenon is called MHC restriction.

5 Fragments of self-proteins are also presented by the APC, but normally such fragments are ignored or not recognized by the T helper lymphocytes. This is the main reason why individuals generally do not harbour autoantibodies in their serum eventually leading to an attack on the indi-
10 vidual's own proteins (the so-called self- or autopro-
teins). However, in rare cases the process goes wrong, and the immune system turns towards the individual's own components, which may lead to an autoimmune disease.

The presence of some self-proteins is inexpedient in
15 situations where they, in elevated levels, induce disease symptoms. Thus, tumour necrosis factor α (TNF α) is known to be able to cause cachexia in cancer patients and patients suffering from other chronic diseases (H.N. Langstein et al. Cancer Res. 51, 2302-2306, 1991). TNF α also
20 plays important roles in the inflammatory process (W.P. Arend et al. Arthritis Rheum. 33, 305-315, 1990) and neutralization of TNF α by the use of monoclonal antibodies has thus been demonstrated to be beneficial in patients with chronic inflammatory diseases such as rheumatoid ar-
25 thritis, Elliott et al., Lancet 1994, 344:1105-10 and Crohn's disease, van Dullemen et al., Gastroenterology 109(1):129-135(1995). There is therefore a need for a method for the induction of neutralizing antibodies against such TNF α proteins, and the present invention
30 comprises a vaccine against TNF α which provide this property.

TUMOUR NECROSIS FACTOR

1. General Background

Tumour necrosis factor (TNF) is a member of the cytokine family of regulatory proteins (see Walsh, G. and Headon, D.E., Protein Biotechnology, 1994, John Wiley & Sons Ltd. England, p. 257-267). Two forms of TNF are now recognized, $\text{TNF}\alpha$ and $\text{TNF}\beta$, respectively. Although both proteins bind the same receptors and elicit broadly similar biological responses, they are distinct molecules and share less than 30% homology. The original protein termed tumour necrosis factor, referred to as TNF, is more properly termed $\text{TNF}\alpha$; it is also known as cachectin. $\text{TNF}\beta$ is also referred to as lymphotoxin.

$\text{TNF}\alpha$ is produced by a wide variety of cell types, most notably activated macrophages, monocytes, certain T lymphocytes and NK cells, in addition to brain and liver cells. The most potent known inducer of $\text{TNF}\alpha$ synthesis, is a complex biomolecule termed lipopolysaccharide. It contains both lipid and polysaccharide components, and is also referred to as endotoxin. Lipopolysaccharide itself is devoid of any anti-tumour activity. The serum of animals injected with lipopolysaccharide was found to contain a factor toxic to cancerous cells, and this factor, produced by specific cells in response to lipopolysaccharide, was termed tumour necrosis factor. Various other agents such as some viruses, fungi and parasites also stimulate the synthesis and release of this cytokine. Furthermore, $\text{TNF}\alpha$ may act in an autocrine manner, stimulating its own production.

Native human $\text{TNF}\alpha$ is a homotrimer, consisting of three identical polypeptide subunits tightly associated around a threefold axis of symmetry as will be further explained below. This arrangement resembles the assembly of protein

subunits in many viral capsid proteins. The individual polypeptide subunits of human TNF α are non-glycosylated and consist of 157 amino acids. The molecule has a molecular weight of 17300 Da and contains a single intrachain disulphide linkage. Human TNF α is synthesized initially as a 233 amino acid precursor molecule. Proteolytic cleavage of the -76 to -1 presequence including a signal sequence releases native TNF α . TNF α may also exist in a 26000 Da membrane-bound form. Three TNF α monomeric subunits associate noncovalently to form a trimer as further explained below.

TNF α induces its biological effects by binding specific receptors present on the surface of susceptible cells. Two distinct TNF α receptors have been identified. One receptor (TNF-R55) has a molecular weight of 55000 Da, whereas the second receptor (TNF-R75) has a molecular weight of about 75000 Da. These two distinct receptor types show no more than 25% sequence homology. TNF-R55 is present on a wide range of cells, whereas the distribution of the TNF-R75 receptor is more limited. Both are transmembrane glycoproteins with an extracellular binding domain, a hydrophobic transmembrane domain and an intracellular effector domain.

The exact molecular mechanisms by which TNF α induces its biological effects remain to be determined. Binding of TNF α to its receptor seems to trigger a variety of events mediated by G-proteins in addition to the activation of adenylate cyclase, phospholipase A₂ and protein kinases. The exact biological actions induced by TNF α may vary from cell type to cell type. Other factors, such as the presence of additional cytokines, further modulate the observed molecular effects attributed to TNF α action on sensitive cells.

The TNF α gene has been cloned and inserted in a variety of recombinant expression systems, both bacterial and eukaryotic. The resultant availability of large quantities of purified, biologically active TNF α has facilitated clinical evaluation a number of diseases, most notably cancer. Many such trials, using TNF α either alone or in combination with interferons, yielded, however, very disappointing results. Large quantities of TNF α can not be administered to patients owing to its toxic - if not lethal - side-effects.

As mentioned above prolonged production of inappropriately elevated levels of TNF α has also been implicated in the development of cachexia, the wasting syndrome often associated with chronic parasitic or other infections, and with cancer. TNF α is also involved in the metastasis and growth of certain tumours as well as in induction of anaemia. Furthermore, TNF α is also directly involved in the development of certain chronic inflammatory disorders in humans, including rheumatoid arthritis and Crohn's disease where administration of monoclonal anti-TNF α antibodies has been shown to be beneficial. TNF α is also involved in osteoporosis and Psoriasis. In addition, it has been shown in animal models that administration of anti-TNF α antibodies may decrease or prevent rejection of grafted or transplanted tissues Imagawa et al, Transplantation 51(1):57-62(1991).

2. Structure of TNF α

I. Introduction

The three-dimensional structure of human tumour necrosis factor (TNF α) has been solved (see "Tumor Necrosis Factors, Structure, Function and Mechanism of Action" edited by Bharat B. Aggarwal and Jan Vilcek, 1992 Marcel Dekker, Ind., New York, Chapter 5 "Crystal structure of TNF α ",

by Jones, E.Y. Stuart, D.I. and Walker N.P.C.). The biological action of TNF α is dependent on its interaction with its receptors. These interactions are governed by the precise arrangement of the correctly folded tertiary structure. Thus, to understand how the TNF α molecule performs its biological function at the level of amino acid interactions, one must not only know the amino acid sequence, but also the three-dimensional structure.

II. Three-dimensional structure

Biologically active TNF α has been shown by analytical ultracentrifugation, small angle x-ray scattering, and gel electrophoresis to be in a trimer conformation in solution, and cross-linking studies have indicated that this is the active form of the protein (Smith and Baglioni, 1987). Analysis of circular dichroism spectra placed TNF α in the all-sheet class of proteins (Wingfield et al., 1987; Davis et al., 1987). Several different crystal forms have been reported for human recombinant TNF α . All the reported crystal forms exhibit crystallographic and/or non-crystallographic threefold symmetry indicative of the presence of TNF α as a trimer within the crystal. The TNF α trimers lie in loosely packed arrays perforated by 100 Å diameter solvent channels. Only a small proportion of the molecular surface is involved in crystal packing contacts. Such contacts could slightly perturb a few side chains and perhaps even short portions of inherently flexible main chain from their preferred solution conformations.

A. Main-Chain Fold of the TNF α Monomer

The overall shape of a single 157-amino-acid subunit of the TNF α trimer is wedgelike with a height of approximately 55Å and a maximum breadth of 35Å near the base. The main-chain topology is illustrated in Fig. 1a-c; it

is essentially a β -sandwich structure formed by two antiparallel β -pleated sheets. The main-chainfold conforms to that of the classic jellyroll motif (Fig. 1c) (Richardson, 1981). The nomenclature adopted in Fig. 1

5 for the labels of the secondary structural units follows the established convention for viral structures. The standard eight β -strands (B to I) are all present but with an insertion between B and C that adds a short strand onto the edge of both β -sheets and truncates the

10 N-terminal half of C, so that each β -pleated sheet contains five antiparallel β -strands, the back β -sheet comprising β -strands B', B, I, D and G and the front sheet comprising β -strands C', C, H, E and F.

The N terminus is highly flexible. This region, as far as

15 residue 10 (see fig. 1b), is rather independent of the rest of the molecule, with the first few residues free to sample a variety of conformations in the solvent. In contrast, the C terminus is embedded in the base of the back β -sheet and forms an integral part of this relatively

20 flat secondary structural unit. The gradation in β -strand lengths and the insertion between β -strands B and C conspire to produce a front surface formed almost entirely of loops, and it is this "masked" side of the β -sandwich, which in the trimer is presented to the solvent. The

25 crystallographic data yield a measure of the relative flexibility of the various parts of the structure. The β -strands form a fairly inflexible scaffold; in particular, the back β -sheet is situated at the core of the trimer and consequently is particularly rigid. As would be expected,

30 it is the loops that adorn the outer solvent-accessible surface of the molecule, which exhibit high levels of flexibility/mobility. Overall, there is a general decrease in rigidity as the core becomes more loosely packed in the upper half of the molecule.

B. General Topology of the TNF α Trimer

Three TNF α monomeric subunits associate noncovalently to form a compact, conical trimer having a length of about 55 Å and a maximum breadth of 50 Å. The β -strands of the three individual β -sandwiches lie approximately parallel (the tilt is about 30°) to the threefold axis of the trimer. The interaction between subunits related by the three-fold axis is through a simple edge-to-face packing of the β -sandwich; the edge of the β -sandwich, consisting of strands F and G from one subunit, lies across the back β -sheet [GDIBB'] of a threefold related subunit (see Fig. 2). The carboxy termini lie close to the threefold axis. The edge-to-face mode of packing produces an extremely tight association between the subunits. Thus the core of the trimer is completely inaccessible to solvent.

C. Amino Acid-Type Distribution

The overall distribution of residue types in the three-dimensional structure of TNF α echoes the general rule for proteins: namely, that hydrophobic residues cluster in the core of the molecule while charged residues decorate the surface. Thus the core of the TNF α sandwich has the expected filling of tightly intercalating large apolar residues.

The energetics of the system do not favour the existence of TNF α in a monomeric state. For a large interface area composed of complementary residues (e.g., polar residues matched against polar residues) the loss of solvent-accessible surface area confers a considerable energetic advantage to formation of the oligomer (i.e., the trimer). The exposure to solvent of the large patch of strongly hydrophobic residues normally buried in the lower portion of the trimeric interface would also act to destabilize the TNF α monomer.

3. Probes of structure-function

A. TNF α /Antibody Interactions

It has been observed that antibodies raised against TNF α from one species (e.g., human) do not cross-react with TNF α from another species (e.g., mouse) despite a sequence identity in excess of 80% and the ability of TNF α to bind to the TNF α receptors of other species. If the degree of sequence variation is mapped onto the three-dimensional structure, it is immediately apparent that the most sequence-variable regions of the molecule correspond to the antibody-accessible surface loops. The regions of highly conserved residues within the sandwich or at the trimeric interface are effectively invisible to antibodies. Thus the epitope for an antibody against TNF α will always contain some residues that will vary between species, thus abolishing antibody binding. This implies that the characteristics of the interaction between TNF α and its receptor must somehow differ from those required for binding of an antibody to TNF α .

B. Site-Directed Mutagenesis

The role of various specific residues and regions of the TNF α molecule with regard to its biological (cytotoxic) activity and receptor binding has been probed by replacement of those residues by different amino acids or deletion of part of the sequence using the techniques of site-directed mutagenesis (Jones et al, p. 113-119).

The deletion of up to eight residues from the N-terminus without any deleterious effect on biological activity serves to emphasize the nonessential nature of this region for overall molecular stability. N-terminal residues appear to exert an indirect, second-order effect on the biological efficacy of the TNF α trimer.

Non-conservative substitutions of the normally highly conserved residues which form the tightly packed core of the β -sandwich distort the structure and hence abrogate the biological activity of TNF α (Yamagishi et al., 1989).

5 Many such mutated proteins fail to form a stable, correctly folded molecule. Some conservative substitutions are permitted within the hydrophobic patch at the bottom of the threefold axis; however, there appears to be much greater leeway in the more loosely packed region near the

10 top of the trimer. In particular, Cys 69 and Cys 101, which form the disulphide bridge between two connecting loops at the loosely packed top of the molecule, are relatively insensitive to changes (see Fig. 1a). Generally, however, in order to retain some biological activ-

15 ity of TNF α the mutations near the central axis of TNF α must be highly conservative, preserving the overall shape of TNF α .

The residues on the surface of the molecule have a considerably greater freedom to mutate without incurring

20 disastrous structural penalties as witnessed by the proliferation of variations of residues in this category between species. Thus drastic reductions in biological activity of TNF α due to substitutions in this area points to the direct involvement of such residues in the func-

25 tional interaction of the TNF α trimer with its receptor. Residues comprising Arg 31, Arg 32 and Ala 33 situated in the connecting loop between the B and B' strand of the back β sheet, Ser 86 and Tyr 87 situated in the connect-

30 ing loop between the E and F strands of the front β -sheet, and Glu 146 situated in the connecting loop between the H strand of the front β sheet and the I-strand of the back β -sheet appear to be such amino acid residues (see Fig.3). They appear to fall into two distinct re-

35 gions on the front and the back sides of the TNF α monomer. The distribution of all deleterious mutations re-

ardless of structural category further reinforces this picture. The existence of these "hot spots" for sensitivity of biological function to mutation has been reviewed by Yamagishi et al. (1989) and Goh et al. (1990).

5 4. Summary

A rich variety of data may now be brought to bear on the specific relationship of structure to function for TNF α . All available evidence points to the importance of the trimer as the stable natural unit. It is apparent that
 10 the two hotspot regions situated on separate sides of the TNF α monomer are brought close to each other in terms of neighbouring subunits in the trimer. Thus a region of functional importance consisting of residues 31 to 35, 84 to 87, and 143 to 148 appears to be located at the inter-
 15 face between two subunits on the lower half of the trimer. Yamagishi et al. (1989) report loss of receptor binding ability as well as cytotoxicity for the mutation of Asp 143 to Tyr, and Tsujimoto et al. (1987) report a similar effect for Arg 31 and Arg 32 to Asn and Thr. Thus
 20 the site may be associated directly with receptor binding as well as cytotoxicity. It is interesting that the receptor binding region of TNF α appears to lie at the interface between two subunits.

In summary, the detailed three-dimensional structure for
 25 TNF α serves to explain a wide range of observations on antibody binding, oligomerization, and site-directed mutagenesis. When the structure is considered in combination with recent, extensive site-directed mutants, a region of biological importance with regard to receptor
 30 binding is apparently at the subunits on the lower half of the trimer.

Description of the prior art

In WO 95/05489 the present inventors disclose a method for the modification of self-proteins so as to induce antibody response against the unmodified self-protein, wherein a self-protein analog is provided by molecular biological means. More specifically one or more peptide fragments of the self-protein are substituted by one or more immunodominant, foreign T-cell epitopes.

It is stated as preferable in WO 95/05489 that the immunodominant T-cell epitope is inserted so that flanking regions from the original protein comprising at least 4 amino acids are preserved on either side of the inserted epitope. In other words the epitope should not be combined with the self-protein as a fusion protein. Apart from that no specific guidance is provided as to the optimal intramolecular position of the inserted epitope in order to create the most powerful antibody response against the unmodified self-protein. Presumably, this will vary from self-protein to self-protein but based on the general guidance in the specification the most appropriate position(s) can be determined without undue experimentation by selecting peptides comprising appropriate immunodominant epitopes, exchanging peptide sequences of essentially the same length in various parts of the self-protein molecule and determining the raised antibody response by suitable assay techniques.

Brief description of the drawings

Figure 1(a) illustrates the crystal structure of native TNF α monomer. The figure is a diagrammatic sketch of the subunit fold, β strands are shown as thick arrows in the amino-to-carboxy direction and connecting loops are depicted as thin lines. The disulfide bridge is denoted by a lightning flash and a region of high flexibility is

crosshatched. The trimer threefold axis would be vertical for this orientation.

Figure 1(b) is a C α chain trace of the TNF α monomer crystal structure. This detailed representation should be
5 used in conjunction with Figure 1(a) to give the precise alignment of the amino acid sequence with the clearer but stylized representation of the subunit fold.

Figure 1(c) shows the TNF α structure as a jelly roll motif. The insertion between β strands B and C is shown in
10 dashed lines; the connection between B and C would run straight across at the top of the molecule.

Figure 2 shows the edge-to-face packing of β sandwiches in the TNF trimer. The view, down the threefold axis, shows a narrow slab of the trimer with β strands represented by ribbons running into and out of the page.
15

Figure 3(a) illustrates the DNA sequence encoding tumor necrosis factor (TNF α) having the amino acid sequence shown in Fig. 3(b)

The DNA sequence is available from Gen Bank under accession no. M10988, SEQ ID NO:339737.
20

The sequence has been described by Wang et al., Science 228, 149-154 (1985). The sequence includes codons encoding the -76--1 presequence of human TNF α .

The complete gene sequence including introns has been described by Nedwin et al., Nucleic Acids, Res. 13(17)
25 6361-6373 (1985), Shirai et al., Nature 313(6005), 803-806 (1985) and Dennica et al., Nature 312 (5996), 724-729 (1984).

Figure 3(b) shows the amino acid sequence of human TNF α including the -76--1 presequence.
30

Figure 4(a) schematically illustrates the substitutions of immunodominant epitopes P2 and P30 in a wild type TNF α (WT) to form the TNF α analogs TNF2-1 to 2-7 and TNF30-1 to 30-5.

- 5 Figure 4(b) shows the exact locations of the substitutions in the WT sequence for the individual TNF α analogs.

Figures 5(a) and 5(b) show the structure of the analogs based on Fig. 1(a), where the individual substitutions by P2 and P30 in the strands of the β -sheets and the connection loops, respectively, are marked in black.

10

Figure 6 shows the biological activity of the TNF α analogs in the L929 assay compared with recombinant TNF α .

Figure 7 shows the anti-human TNF α antibody response in rabbits to vaccination with the modified TNF α molecules in rabbits.

15

Figure 8 shows the ability of P2/P30 modified human TNF α molecules to induce neutralizing antibodies as measured in the L929 cell assay.

Figure 9 shows the ability - when administered to rabbits - of P2/P30 modified human TNF α molecules to induce neutralizing antibodies as measured in receptor assay.

20

Figure 10 shows the Peripheral Blood Mononuclear Cell (PBMC) response in three donors towards TT and the P2 and P30 peptides.

25 Figure 11 shows the polyclonal proliferation response in two donors using the different P2 and P30 modified TNF α molecules.

Figure 12 shows the Proliferation Indexes (PIs) calculated from 34 experiments for the P2 and P30 modified TNF α molecules.

Figure 13 shows the PBMC response against P2 and P30 modified TNF α proteins in P2 and P30 specific responders, respectively.

Figure 14 shows a similar PBMC response in two other donors.

Figure 15 shows the influence of flanking amino acids on the T cell recognition of P2 and P30.

Figure 16 shows the mutation strategy used for the preparation of the modified TNF α molecules.

Summary of the invention

The purpose of the present invention is to provide guidelines as to how a particular self-protein within the general scope of the above-mentioned WO 95/05489, viz. human TNF α should be modified in order to be biologically inactive as well as be able to induce a strong neutralizing antibody response towards unmodified, biologically active TNF α . In the present context "biologically inactive" refers to the activities of the unmodified TNF α , mainly its cytotoxic activity.

From the discussion of the tertiary structure of TNF α given above, it is recalled that the biologically active TNF α is a trimer of three subunits. Due to the "edge-to-face" packing the "back β -sheet" represents the "hidden" area of contact between the subunits which is completely inaccessible to solvent. Significant substitutions in this area will almost inevitably deprive the TNF α molecule of all biological activity. On the contrary the "front β -sheet" and the connecting regions provide the

accessible surface area which includes the areas interacting with the TNF α receptors. Antibodies towards these areas would therefore probably be able to interfere with receptor binding and would hence possess TNF α neutralizing properties.

A person skilled in the art who wanted to construct a detoxified and yet immunogenic TNF α molecule according to WO 95/05489 would therefore as the first choice insert the immunodominant T cell epitope in the back β -sheet of the TNF α monomer. Modifications of this area would thus most probably interrupt the biological activity of TNF α and leave the receptor-accessible front β -sheet free for interaction with antibodies. This is also consistent with the discussion of the site-directed mutagenesis in the tightly packed core of the β -sandwich discussed above.

However, surprisingly it is not so. As it will appear from the test results below, the result was quite the contrary, since substitutions comprising the B and G strands of the back β -sheet surprisingly provided TNF α analogs which were unable to induce neutralizing antibodies against TNF α . On this background the modified human TNF α molecules according to the present invention are characterized in that the substitution has been made in regions of the TNF α molecule which do not comprise the B and G strands of the back β -sheet of the molecule.

Although it has not yet been fully verified experimentally, it must be assumed that this property is common to the strands forming the back β -sheet, so that preferably the substitutions should be made in regions of the TNF α molecule which do not comprise any strand of the back β -sheet. In view of the discussion above of the functional importance of the residues 31-35 it can be assumed that

the connecting loops between the individual strands of the back β -sheet should preferably also be avoided.

However, it is permissible that the substitution is made in regions of the TNF α molecule which only involve a segment of the D strand of the back β -sheet.

According to a preferred embodiment of the invention the substitution comprises at least a segment of the H strand of the front β -sheet and of the connecting loop to the I strand of the back β -sheet, preferably amino acids 132 to 146. According to another embodiment of the invention the substitution comprises segments of the H and I strands and the entire connecting loop, preferably amino acids 132 to 152. According to yet another presently preferred embodiment of the invention the substitution comprises a segment of the D strand of the back β -sheet, at least a segment of the E strand of the front β -sheet and the entire connecting loop, preferably amino acids 65 to 79 or 64 to 84.

According to a further embodiment of the invention the substitution comprises the entire C' and C strands of the front β -sheet and a segment of the D strand of the back β -sheet, preferably amino acids 40 to 60.

According to a still further embodiment of the invention the substitution comprises at least a segment of the E strand of the front β -sheet and of one or both of the connecting loops, preferably amino acids 76 to 90.

The inserted T cell epitope should preferably be promiscuous and known to be immunogenic in a majority of human HLA class II types. Applicable epitopes can be derived e.g. from Tetanus toxoid, preferably epitope P2 and/or P30, Panina-Bordignon et al., Eur. J. Immunol. 19:2237-

42, 1989. Also epitopes derived from diphtheria toxoid may be used.

The preferred modified human TNF α molecules (TNF α analogs) as referred to above with reference to the location of the substitution are shown in the enclosed sequence listing as SEQ ID NO:8 and SEQ ID NO:16.

Other applicable TNF α analogs are listed as SEQ ID NO:4, 10, 14 and 16.

The invention also relates to truncated analogs of the above-mentioned modified TNF α analogs according to the present invention. Thus, truncated analogs of TNF α molecules containing a promiscuous and immunodominant T cell epitope and one or both flanking regions comprising at least one TNF α B cell epitope, preferably comprising at least five amino acids, would also constitute a possible TNF α vaccine according to the invention. The T cell epitope would induce the proliferation of T cells when presented to MHC class II molecules by the APC, while B cell epitope would potentially be recognized by the immunoglobulin receptors on B cells, and would subsequently be presented by MHC class I molecules on these cells. This constitutes the basis for raising an immune response towards native TNF α , harbouring the B cell epitope, according to the WO 95/05489 and the present invention.

B cell epitopes could be identified in the TNF α both theoretically and experimentally. Algorithms for identification of potential linear B cell epitopes have been published, and this would form the basis of an experimentally based investigation of the nature of these potential epitopes. Antibodies raised in a heterologous system (e.g. rabbits) in response to injections of such truncated TNF α molecules comprising T cell epitopes could be analyzed for *in vitro* capability to bind native human

TNF α , preferentially in neutralizing manner. Panels of monoclonal antibodies known to be neutralizing could be screened *in vitro* for capability to bind the potential B cell epitopes of TNF α . Both of these are strategies for
5 identifying the possible and best B cell epitopes.

The invention further relates to dimers, oligomers, especially trimers or multimers of the claimed modified TNF α molecules and isolated DNA molecules that code for the claimed modified TNF α molecules.

10 The isolated DNA molecules encoding the preferred TNF α analogs have the sequences listed as SEQ ID NO:7 and 15, and the DNA molecules encoding the other applicable analogs are listed as SEQ ID NO:3, 9, 13 and 15.

The invention further comprises vectors comprising the
15 isolated DNA molecules encoding the analogs and expression vectors comprising said DNA molecules operatively linked to a suitable expression control sequence.

Another aspect of the invention is a host transformed with an expression vector for said analog.

20 Said host may be any of the hosts commonly used for expression, e.g. a strain of bacteria, yeast or fungi or insect, mammalian or avian cell lines.

The invention further relates to a method of producing the claimed TNF α analogs, whereby host cells transformed
25 with an expression vector for the analog is grown under suitable conditions permitting production of the analog, and the produced analog is recovered.

If desired, the modified TNF α molecules according to the invention may be expressed as or form part of a fusion
30 protein with a suitable adjuvant molecule, preferably an

immunologically active adjuvant, such as GM-CSF, HSP70 or an interleukin, e.g. interleukin 2.

More specifically, the modified TNF α molecules are produced by substituting the appropriate gene segments encoding the immunodominant T cell epitopes into the gene encoding the native human TNF α molecule. Subsequently the modified TNF α gene is expressed in an appropriate eukaryotic or prokaryotic expression vector. The expressed modified TNF α molecules are purified and refolded as described below.

According to the invention the modified human TNF α molecules may be used in vaccines against TNF α . Usually vaccines require the use of adjuvants. According to the invention the modified human TNF α molecules can be formulated with such appropriate adjuvants, e.g. aluminium phosphate (Adju-Phos) or other alternative adjuvants such as aluminium hydroxide, calcium phosphate, muramyl dipeptide analogs, Iscom's or other known adjuvants used in mammalian vaccines.

The vaccines may be directed against any of the TNF α -dependent diseases described above, in particular chronic inflammatory diseases. As examples can be mentioned rheumatoid arthritis and inflammatory bowel diseases (IBD). The latter includes ulcerative colitis and Crohn's disease, in particular Crohn's colitis. Other examples are cancer, cachexia, often related to cancer, disseminated sclerosis, diabetes, psoriasis, osteoporosis and asthma. Preferably the vaccines will be given as a preventive treatment, but in view of the chronic nature of these diseases and their tendency to remission and recurvency, they may also be administered to patients, where one or more of the above-mentioned diseases has been diagnosed,

and may serve to maintain the patient in a state of remission.

Based on earlier studies in mice, it is believed that the modified TNF α analogs according to the invention can also
5 be administered as part of a curative treatment of the above-mentioned diseases in an acute state or at least with a view to bringing the patient in remission and maintain a steady state condition.

At present no specific effective dose range can be
10 stated, since the vaccines have not yet been tested in human beings susceptible to any of the diseases.

At any rate the administered dose will be prescribed by the responsible doctor.

According to one embodiment of the invention the vaccine
15 comprises a mixture of two differently modified TNF α molecules containing two different T cell epitopes e.g. P2 and P30 which are derived from tetanus toxoid. This mixture optionally contains appropriate amounts of pharmaceutically adjuvant

20 According to yet another aspect of the invention, the vaccines do not comprise the modified human TNF α molecules as such, but rather a construct comprising non-infectious non-integrating DNA sequence encoding said molecules operatively linked to a promoter sequence which
25 can control the expression of said DNA sequence in humans, in an amount sufficient that uptake of said construct occurs, and sufficient expression occurs to induce a neutralizing antibody response against TNF α .

The utility of this type of vaccines, the so-called DNA
30 vaccines, is illustrated e.g. in US patents nos. 5.589.466 and 5.580.859, both of which are incorporated

herein by reference, in particular in relation to the methods of administration.

The DNA vaccines may comprise a viral expression vector, such as a retroviral expression vector.

- 5 Generally, the vaccines according to the invention may be adapted for oral or parenteral, in particular subcutaneous, intramuscular or intradermal administration.

The invention further comprises the use of antibodies raised by administering a vaccine according to the invention, preferably monoclonal antibodies, particularly a
10 diagnostic use.

The invention further relates to a method of testing human body fluids for the presence of TNF α which comprises contacting a composition containing modified TNF α according to the invention with a sample of human body fluid
15 and determine, whether said antibodies bind to TNF α in said sample.

The invention also relates to a diagnostic method for TNF α -related diseases employing an in vitro immunoassay
20 to detect TNF α in human body fluids.

Said methods may involve the use of a sandwich assay, ELISA assay or equivalent assay, which can be unamplified or amplified, e.g. using avidin/biotin technology.

25 EXPERIMENTAL PART WITH DESCRIPTION OF PREFERRED EMBODIMENTS

1. Introduction

In the previous application WO 95/05489 it was shown that T cell epitope modified murine TNF α molecules could induce high titers of antibodies cross-reactive with native

murine TNF α . These antibodies were able to interfere with TNF α and its receptor *in vitro* as well as *in vivo*. Beneficial effects of immunization against TNF α were demonstrated in several animal models of TNF α -induced disease such as experimental cachexia, collagen arthritis and experimental allergic encephalomyelitis (EAE). These animal experimental results were obtained despite the fact that the two modified murine TNF α molecules used (denominated MR 103 and MR 106) were not optimized to be immunogenic in the MHC class II haplotypes of DBA/1 and SJL mice. These mouse strains were used for the collagen arthritis and the EAE experiments, respectively.

MR 103 and MR 106 were mouse molecules and based on the prior application WO 95/05489 no specific conclusions could be drawn with regard to the immunogenicity of appropriately T cell substituted human modified TNF α molecules nor about the potential ability of such molecules to induce TNF α neutralizing autoantibodies.

2. Development of human TNF α constructs

In general terms in a TNF α vaccine for human use the modified human TNF α molecules should fulfill the following requirements:

- a. They should be immunogenic in a large proportion of the population
- 25 b. They should be optimally able to induce TNF α neutralizing antibodies
- c. They should not possess any remaining biological TNF α activity

Furthermore, in a selection process other practical parameters such as levels of recombinant expression, ease

of purification, solubility etc. could also be considered.

2.1. Immunological promiscuity of the modified TNF α molecules

5 During the development of the human TNF α vaccine the aim was to produce modified human TNF α molecules which eventually will be immunogenic in the largest possible part of the human population which of course represents a large number of different HLA class II types. Therefore,
10 instead of the MHC specific epitopes used in the previous animal experiments, promiscuous T cell epitopes were used. It was not known from the previous application WO 95/05489 how such epitopes could influence the capability of such molecules to induce neutralizing antibodies.

15 The two tetanus toxoid (TT) derived T cell epitopes, P2 and P30, which have been well characterized in the scientific literature were chosen. These epitopes are known to be immunodominant in TT and to be able to bind to at least 80 % of the HLA class II molecules in the human
20 population.

Furthermore, by using these TT epitopes it was expected to be possible to test the immunogenicity of the TNF α constructs *in vitro* on peripheral blood mononuclear cells (PBMC) and T cell lines generated from TT immune blood
25 donors.

The amino acid sequence of the P2 epitope is QYIKANSKFIGITEL and corresponds to TT amino acids 830-844, and the sequence of the P30 epitope is FNNFTVSFWLRVPKVSASHLE and corresponds to TT amino acids
30 947-967. Substituting P2 and P30 into two different human TNF α molecules would exchange approximately 10 % and 15 %, respectively, of the native TNF α sequence. In case

both epitopes were inserted into a single TNF α molecule, about 25 % of the molecule would be exchanged, and one could fear that this would interfere too much with the remaining native parts of the TNF α molecule. It was therefore decided to develop two TNF α molecules, each containing either P2 or P30. Together, such two molecules would be expected to be immunogenic in at least 80 % of the human population. In addition, it is very likely that truncated molecules composed partly of the P2 or P30 epitope and partly of TNF α flanking regions also will contribute to the immunogenicity resulting in the constructs being immunogenic in almost 100 % of the population.

Although it was possible to induce antibodies with all murine TNF α constructs in all mouse strains tested so far, one would *a priori* expect, that insertion of the foreign T cell epitope at certain positions in TNF α would be more beneficial than other positions with regard to the presentation of the epitope to T cells by MHC class II molecules. It was therefore decided to produce an array of differently modified human TNF α molecules with the P2 and P30 epitope inserted at different positions in the molecule, see fig 4.. Subsequently, all molecules were tested *in vitro* in T cell assays based on peripheral blood mononuclear cells (PBMC) or P2/P30 specific T cell lines isolated from a number of healthy TT immune blood donors.

Contrary to what was expected, however, it was shown that although minor quantitative differences were seen, the intramolecular position of the P2 and P30 epitopes was not essential for the ability to be processed by antigen presenting cells and subsequently presented to TT specific T cells. Thus, P2 inserted in positions 132 to 146, 65 to 79 and 76 to 90 and P30 inserted in positions 40 to 60 and 132 to 152 (TNF2-5, 2-3, 2-7, 30-2, 30-5) were all

processed and presented to T cells. So from the discussion above it is very likely, that these molecules eventually will be universally immunogenic in the human population.

5 2.2. The ability of the modified human TNF α molecules to induce neutralizing antibodies

As mentioned above, it was not possible from the previous mouse studies described in WO 95/05489 to predict which position would be most appropriate in order to be able to
10 induce neutralizing TNF α antibodies. An array of different human TNF α molecules with P2 or P30 inserted at different positions was therefore produced. The substitutions were randomly distributed over the entire molecule. The antibodies induced in rabbits upon injection of these
15 molecules were subsequently tested in biochemical as well as biological *in vitro* assays for their ability to interfere with TNF α biological activity.

It has been shown in non-published observations by the present inventors that depending on the intramolecular
20 position of the inserted epitope a different overall specificity of the induced autoantibodies was observed. Quite contrary to what would be expected based on the structural data of the TNF α molecule it was observed, that substitutions in the front β -sheet with either P2 or
25 P30 totally deprived the molecules of biological TNF α activity, but at the same time preserved the ability of the modified molecules to induce TNF α neutralizing antibodies.

The molecules containing P2 or P30, in the positions mentioned above, were shown to be particularly effective at
30 inducing neutralizing antibodies. Any of these molecules are therefore potential candidates for use in human TNF α vaccines.

2.3 The biological activity of the different TNF α constructs

It would obviously not be feasible to use a molecule which is as toxic as TNF α in a vaccine. The modified TNF α molecules would therefore have to be non-toxic i.e. devoid of any residual TNF α activity.

All mutant TNF α proteins were therefore tested *in vitro* in TNF α dependent bioassays as well as receptor binding assays in order to examine whether they are non-toxic. It was shown clearly that the modified human TNF α molecules (TNF2-5, 2-3, 2-7, 30-2, and 30-5,) all were deprived of TNF α biological activity. So all the necessary requirements of these molecules to be part of a universally, non-toxic vaccine capable of inducing anti-human TNF α neutralizing antibodies were fulfilled

EXAMPLE 1Genetic construction work

It was decided to produce 10 different modified human TNF α molecules - five containing the P2 and five containing the P30 epitope. The epitopes were distributed at different positions within the molecule. The genetic constructions were made by using various standard restriction enzyme and PCR based mutagenesis techniques. The genetic constructs are shown schematically in Fig. 4a and 4b. DNA sequences encoding the modified TNF α molecules and the corresponding amino acid sequences are incorporated as SEQ ID NO:1 - SEQ ID NO:20. The constructions of the mutant human TNF2-5 gene, the cloning and mutation strategy, and the subsequent expression, isolation and purification of the TNF2-5 analog is explained below by way of example.

Construction and production of TNF2-5:Genetic construction of the mutant human TNF2-5 gene, cloning and mutation strategy.

The genetic construction of the gene encoding the mutant human TNF2-5 analog was based upon traditional PCR based mutagenesis techniques, as were all the other genetic constructions.

The native DNA sequence of human TNF α encoding the soluble part of this molecule was obtained by traditional PCR cloning using synthetically synthesized primers I and II (table 1 and SEQ ID NO:21 and 22) from a human commercially available cDNA library, CLONTECH Laboratories, Palo Alto, CA, USA (Fig. 16,1). The native gene was inserted into a commercial *E.coli* expression vector pET28c available from Novagen, Madison, WI 53711, USA, in such a

way that the gene could be transcribed in frame from an IPTG inducible promoter.

The genetic construction of the TNF α mutant analog TNF2-5 was performed by a PCR mutagenesis technique applied to the native DNA sequence. The nucleic acid sequence encoding the T cell epitope was incorporated into a synthetically synthesized 75-mer' oligonucleotide (Primer "mut2-5", table 1 and SEQ ID NO:27) between the two 3'- and 5'-annealing stretches of TNF homologous DNA, the "mut" primer is thus capable of annealing to the native human TNF α gene sequence at the defined site selected for TNF2-5, (see Fig 16,2a). In the "mut" oligonucleotide the number of codons encoding the T cell epitope exactly matched the number of TNF-codons omitted between the two 3'- and 5'- annealing stretches of TNF homologous DNA. The mutagenesis primer was used to produce a PCR product containing the DNA encoding the T cell epitope and the TNF α sequence below (or 3') to the inserted epitope, (Fig. 16, 2a). The stretch of TNF α DNA above (or 5') to the point of insertion of the epitope was provided by a second PCR product using primers I and III (Table 1, SEQ ID NO:23) (Fig. 16, 2b). The two PCR products are eventually joined together in a final PCR reaction, (Fig. 16, 3) using the two most distal primers, (I, II) from the two reactions. The complete mutant TNF2-5 DNA sequence is then introduced into a commercial *E.coli* expression vector in analogy to the expression cloning of the native gene in such a way that the gene could be transcribed from an IPTG inducible promoter from transformed cells.

The "mut" primers used for construction of the other analogs (TNF2-1, 2-3, 2-4, 2-7, 30-1, 30-2, 30-3, 30-4 and 30-5) are identified as SEQ ID NO:23-26 and 28-33, respectively.

Table 1

	Primer I	HumanTNF-alpha FW.	24'-mer.
	NcoI-site		
	5'-GAC AAG CCC ATG GTC AGA TCA TCT-3'		
5	Primer II	HumanTNF-alpha Rev	30'-mer.
	XbaI-site.		
	5'-TCT CTA GAG GGC AAT GAT CCC AAA GTA GAC-3'		
10	Primer "mut2-5"	Mutant oligo" P2-5 (tt830-44),	75'-mer.
	5'-G AAG GGT GAC CGA CAG TAC ATT AAG GCC AAT TCG AAG TTC ATT GGC ATC ACT GAG CTG TCT GGG CAG GTC TAC TT-3'		
	Primer III	HumanTNF-alpha Rev 2'nd.	21'-mer.
15	5'-CCC AAA GTA GAC CTG CCC AGA-3'		

Cultivation of Recombinant Bacteria, harvest and dissolve inclusion bodies.

Protein purification of the TNF2-5 analog

20 The production of the TNF2-5 protein was analogous to the production of the other recombinant TNF molecules.

1. Inoculate 20 ml TB medium containing 50 µg/ml Carbenicillin with the transformed *E.coli* strain carrying the IPTG inducible plasmid vector harbouring the TNF gene encoding the recombinant protein, grow the *E.coli* over
25 night at 37°C with shaking.

2. Dilute the over night culture 1:25 in 250 ml TB medium with 50 µg/ml Carbenicillin, and grow the culture until OD₄₅₀ is 1. Induce the expression of the recombi-
30 nant protein by adding IPTG to a final concentration of 1 mM. Grow over night with vigorous shaking at 37 °C.

3. Harvest the recombinant cells from the medium by centrifugation at 3500 x g. Wash the pellet once in BSB buffer. Use 150 ml BSB per 50 g wet weight bacteria.
4. Sonicate 4 times 30 seconds at maximal amplitude until the bacterial suspension is completely homogeneous. The sonication is performed using a MSE Soniprep 150 sonicator mounted with a 9.5 mm *standard probe* (Soniprep 05 38 121-1154)
5. Add 8 μ l PMSF (50 mM) and 80 μ l lysosyme solution (10 mg/ml) per gram of cell pellet. Incubate 30 min at RT
6. Add 4 mg deoxycholic acid per gram pellet, mix and store at 37 °C
7. When the solution has become viscous add 20 μ l DNase (1 mg/ml) per gram pellet and $MgCl_2$ to a final conc. of 5 mM, mix and store at room temperature for 30 min.
8. Sonicate in ice 5 times 30 seconds with 30 seconds intervals at maximum amplitude, until the solution has become fluid and non-viscous.
9. Centrifuge at 20.000 x g for one hour, preserve the supernatant for later checking of the washing procedure to check if all the inclusion bodies have been precipitated.
10. Resuspend the pellet in MiliQ water (1 ml H_2O per gram of E. coli), shake 1 hour.
11. Centrifuge at 20.000 x g for one hour, preserve the supernatant to check if all the inclusion bodies have been precipitated.

12. Resuspend the pellet in 1 M urea dissolve in 1 ml per gram of E. coli, shake 1 hour.
- 13 Centrifuge at 20.000 x g for one hour, preserve the supernatant to check if all the inclusion bodies have
5 been precipitated.
- 14 Resuspend the pellet in 1 M guanidine dissolved in 1 ml per gram of E. coli, shake 1 hour.
- 15 Centrifuge at 20.000 x g for one hour, preserve the supernatant to check if all the inclusion bodies have
10 been precipitated.
- 16 Resuspend the pellet in 25 ml 6 M guanidine + 20 mM Tris pH 8.0, agitate over night.
- 17 Centrifuge at 20.000 x g for one hour, preserve the supernatant containing the recombinant protein inclu-
15 sion bodies, preserve the pellet to check if all the inclusion bodies have been dissolved
- 18 The protein solution is extensively dialyzed against MilliQ water, and subsequently the solution is freeze dried.
- 20 19 The freeze dried material is solubilized in 20 mM Tris, 6 M guanidine, 30 % 2-propanol (pH 8.0) at a concentration of 20 mg/ml. It is allowed to solubilize overnight under gentle agitation. The presence of mono-
mers is examined on a superdex 200 column (XK 16, Pharmacia, diameter: 1.6 cm, height: 750 cm.). Run in running
25 buffer at 1 ml/min. Compared to standards in the same buffer.
- 20 Protein purification is performed by gel filtration on a superdex 200 column (XK26, Pharmacia; height:
30 100 cm, diameter: 2.6 cm) which is equilibrated with

equilibration buffer. Run in the equilibration buffer. A sample volume of about 1% of total column volume is applied.

21 Refolding of the recombinant protein is performed by dialysis. The protein is diluted to 0.1 mg/ml in equilibration buffer, and this solution is placed in a boiled dialysis bag and dialyzed against: 20 mM Tris, 4 M urea (pH 8.5) with three changes, one night over at room temperature. The dialysis bag is transferred to a Tris buffer (20 mM Tris, 150 mM NaCl (pH 8.0)). Change three times of which the first one takes place at room temperature. Overnight in the cold room.

Refolding is evaluated on a Superose 12 column equilibrated in Tris buffer (20 mM Tris, 150 mM NaCl (pH 8.0)). Compare to standards.

Storage. The recombinant proteins are stored freeze dried.

TB medium

20 Dissolve Terrific Broth (GIBCO BRL 22711-22) in MiliQ water according to the manufacturers instruction. Autoclave at 121 °C for 20 min.

Carbenicillin x 100 stock solution (50mg/ml)

25 Carbenicillin disodium salt (Sigma C1389) is dissolved in MiliQ water at a concentration of 50 mg/ml. The solution is filtersterilized through a 0.2 µm filter (Sterifix 0409 9206)

IPTG x 100 stock solution 100mM

Isopropyl-beta-D-thiogalactopyranoside (IPTG, USB 17884)
1.19 g IPTG is dissolved in MiliQ water ad 50 ml. The so-
5 lution is filtersterilized through a 0.2 μ m filter
(Sterifix 0409 9206)

BSB buffer10 **Bacterial Suspension Buffer**

50 mM TRIS (Trisma base, SigmaT1503)

0.5 M NaCl (Ridel-de.Haën 31434)

5 mM DTT (DL-dithiothretiol, Sigma D-0632)

pH 8.0

15

PMSF

50 mM, phenylmethysulfonylfluride, SIGMA # P-7626,
dissolved in 2-propanol

20

Lysosyme solution

10 mg/ml Grade III lysozyme from chicken egg white, (EC
3.2.1.17) SIGMA # L-7001

25

Deoxycholic acid

(7-deoxycholic acid) Sigma # D-6750

30 **DNase**

1 mg/ml Dnase I, Deoxyribonuclease I, (EC.3.1.21.1) Boe-
hringer Cat # 1284932

UREA

Urea (GibcoBRL 15716-020)

5 Guanidine

Guanidine hydrochloride (Sigma G4505)

2-Propanol

10

Running buffer

20 mM TRIS, (Trisma base, SigmaT1503)

8 M urea, (GibcoBRL 15716-020)

15 0.1% β -mercaptoethanol

pH 8.0

Equilibration buffer

20 20 mM TRIS, (Trisma base, SigmaT1503)

8m urea, (GibcoBRL 15716-020)

0.1% β -mercaptoethanol

EXAMPLE 2Expression, purification and refolding of P2 and P30 modified TNF α molecules

It is well established that recombinant proteins behave
5 differently during expression, purification and refold-
ing. All proteins were expressed in *E. coli* and expres-
sion levels ranging from 2-20 % were obtained. All the
proteins were recognized in Western blotting experiments
using a commercially available polyclonal rabbit-anti hu-
10 man TNF α antibody.

The TNF α constructs were subsequently expressed one by
one in 250 ml cultures in batch sizes of 3-4 l. All modi-
fied TNF α proteins were expressed as inclusion bodies and
more than 85 % pure and refolded protein preparations
15 were produced as described above.

The protein content was determined with standard BCA
analysis. All protein purifications were performed in at
least three separate runs for each molecule, and in all
cases the separate protein batches were tested and found
20 similar. The proteins were stored in concentrations from
0.2 - 1.0 mg/ml in PBS at -20 °C until use.

Standard quality control analyses included SDS gel elec-
trophoresis with both Coomassie blue staining and silver
staining of the individual protein preparations. In all
25 cases the proteins were found to be of the expected size
and to be more than 85 % pure.

Molecules with epitopes inserted at position 4 (TNF2-4
and TNF30-4) were only expressed at relatively low levels
(app. 2 %). These molecules were furthermore very diffi-
30 cult to purify and especially their refolding was trou-
blesome. Both molecules, but in particular TNF30-4, were

not very soluble in PBS and tended to precipitate during storage.

EXAMPLE 3.

Biological activity of the different TNF α constructs

5 The direct biological activity of the purified TNF α molecules was tested in the L929 bioassay which is a standard *in vitro* assay for determination of biological TNF α activity. As seen in Fig. 6 none of the P2 or P30 modified TNF α molecules were able to kill L929 cells in the concentration range tested (up to 60 mg/ml). A commercially available recombinant TNF α molecule were fully active at
10 about 0.6 ng/ml. The wild type TNF α preparation was also fully active in the entire concentration range tested.

EXAMPLE 4.

15 The ability of the modified TNF α molecules to induce neutralizing antibodies

Rabbits were immunized with each of the ten constructs to see whether these were able to induce antibodies capable of neutralizing biologically active human TNF α *in vitro*.
20 Groups of three rabbits were used and each rabbit received 100 mg TNF α s.c. in Freund's Complete Adjuvant and subsequently 100 mg approximately every third week in Freund's Incomplete Adjuvant.

During the entire immunization period of 18 weeks blood
25 samples were collected at regular intervals and the sera were tested for anti-TNF α activity in a conventional ELISA assay where commercially available, pure and non-modified human TNF α was used as the antigen.

All rabbits developed anti-TNF α antibodies during the immunization period and the maximum level of antibody
30

titers varied within a decade within each group. The average antibody titers are shown in Fig. 7. TNF2-5, TNF2-7, TNF2-3, TNF2-1 and WT-TNF α induced a titer of one hundred within 2-4 weeks whereas TNF2-4 gave a remarkably slower response. Similar kinetics were observed for the TNF30 proteins where a slower response also was obtained with TNF30-4.

The ability of these sera to interfere with human TNF α and its receptor was tested in the L929 assay as well as in a solid phase receptor binding assay.

In the L929 assay dilutions of immune sera as well as a non-immune control serum were added to commercially available human TNF α prior to the addition of the mixture to L929 cells. Sera from all three rabbits in each group were tested in duplicates and the average values were calculated. Since normal serum tended to increase the background values of the color detecting system these were subtracted from all values and the relative inhibition in percent was calculated. The results for the inhibitory capacity at week 14 in the immunization schedule of all rabbits are shown in Fig. 8.

TNF2-5 which does not comprise substitutions in any segment of the back β -sheet strands is clearly superior compared to the other constructs and this molecule was comparable to the fully toxic WT-TNF α molecule with regard to the ability to induce neutralizing antibodies (data not shown). TNF2-3 which comprises a small substituted segment in the D β -strand and TNF2-7 which does not comprise any substituted segment of the back β -sheet strands, were also able to induce inhibitory antibodies, and the neutralizing antibody titer towards TNF2-7 increased significantly during the following three weeks (data not shown). TNF2-4 and TNF2-1, which comprise the G

and B β -strands, respectively, of the back β -sheet, were unable to induce neutralizing antibodies in the L 929 assay.

5 The results with regard to the TNF30 proteins were more heterogeneous although TNF30-3 seemed to be best at week 14. TNF30-1 and TNF30-4, which comprise substitutions in the G and B β -strands of the back β -sheet, respectively, did not induce neutralizing antibodies in the L929 assay.

10 In the solid phase receptor binding assay recombinant human 55 kD TNF α receptor 1 (TNF-R55) was immobilized on microtiter plates and commercially available biotinylated human TNF α was added in an appropriate dilution. Specific binding to the receptor was subsequently detected with
15 horse radish peroxidase labeled streptavidin and a chromogenic substrate. When testing sera from immunized rabbits these were added to the biotinylated human TNF α solution prior to the addition of the mixture to the TNF-R55 coated microtiter plate. Sera from all three rabbits in each group were tested and the average values
20 were calculated. Serum from a non-immunized rabbit was used as negative control. The background values were very low and the assay is highly sensitive. The results are shown in Fig. 9.

25 It can be seen that the results obtained from the L929 assay and the solid phase receptor binding assay, respectively, are almost identical with regard to the TNF α 2 constructs. In the solid phase assay the difference between TNF30-2, 30-3 and 30-5 was not as pronounced as observed in the L929 assay. The solid phase assay is, however,
30 more reproducible due to its biochemical rather than cellular character, and normal serum values were not subtracted in this assay.

The relative amounts of serum (in percent) by which half maximum inhibition of TNF α binding was achieved (IC₅₀ values) were calculated for TNF2-3, TNF2-5, TNF2-7, TNF30-2, TNF30-3, TNF30-5 and WT-TNF α for each of the
5 corresponding antisera. Assuming that a similar curve shape would appear for antisera raised against TNF2-1, TNF2-1, TNF30-1 and TNF30-4 extrapolations were performed, and IC₅₀ values were also calculated for these sera. The results are shown in Table II.

Table II.

IC50 values of rabbit-anti human TNF α sera in the solid phase assay										
2-1	2-3	2-4	2-5	2-7	WT	30-1	30-2	30-3	30-4	30-5
>100%	2.02%	>100%	0.53%	1.38%	0.43%	>100%	0.82%	0,88%	>74%	1.69%

It can be seen that TNF2-5 is equivalent to wild type (WT) mouse TNF α with regard to the ability to induce neutralizing anti-mouse TNF α antibodies in rabbits. TNF2-1, TNF2-4, TNF30-1 and TNF30-4 which all comprise substitutions in the B or G strand of the back β -sheet are very poor or even totally unable to induce such antibodies. This indicates surprisingly that although the strands B and G of the back β -sheet are not involved in receptor binding, the mutations represented in this area anyhow lead to disturbance of the regions of human TNF α involved in receptor binding. TNF30-2 and TNF30-3 seem to induce neutralizing antibodies equally well.

EXAMPLE 5.

The ability of the modified TNF α molecules to stimulate T cells from P2 and P30 immune healthy blood donors

Since it was expected that the localization of the P2 and P30 epitopes in the modified TNF α molecules also would affect the antigen processing and presentation of the inserted epitopes - and thus their immunogenicity - all 10 molecules were tested in different T cell assays. A polyclonal Peripheral Blood Molecular Cell (PBMC) assay was used, and a number of P2 and P30 specific T cell lines were established using conventional immunological methods to test TNF α peptides and recombinant proteins with inserted P2 and P30 epitopes.

Initially 28 healthy volunteers (donors) were tested in a conventional PBMC proliferation assay for their ability to respond to TT and the P2 and P30 peptides. On the basis of these results 19 individuals capable of responding significantly to TT, P2 as well as P30 peptides were selected for further experiments. Although the response levels varied much, this clearly confirmed that P2 and

P30 are promiscuous as well as immunodominant T cell epitopes.

In addition to the 28 donors, seven further donors were also selected. For reasons explained below some of these were selected for their ability to respond either to P30 or to P2. Several of these were furthermore vaccinated with TT in order to be able to give a strong T cell response. Some of the second group of donors were also used to raise P2 or P30 specific T cell lines in order to study the antigen presentation of P2 and P30 modified synthetic TNF α peptides as well as the modified molecules. In Fig. 10 representative examples of the polyclonal PBMC proliferative response in three donors towards TT as well as the P2 and P30 peptides are shown.

All 10 recombinant proteins were tested in triplicate in at least 6 different concentrations starting from about 100 mg/ml, and all PBMC experiments were repeated 2-3 times for each individual. Intracellular incorporation of ^3H -labelled thymidine was used to assess T cell proliferation, and the experiments were harvested and counted in a 96-well format. The maximum proliferation indexes (PI) from each titration curve was calculated as the relation between the average number of CPMs in the experimental wells divided by the average number of CPMs obtained in the antigen free wells (PBS only). The T cell proliferation data were made in triplicate and Con A as well as the P2 and P30 were used as negative and positive controls, respectively. In Fig. 11 three examples are shown from experiments with two different blood donors using the different P2 and P30 modified TNF α molecules.

JH only responds to P2, whereas SR responds to both P2 and P30. This is also reflected in the proliferative responses to the P2 and P30 modified TNF α proteins which to

a varying extent almost all are able to stimulate the PBMCs.

In Fig. 12 the Proliferation Indexes (PIs) calculated from 34 experiments are shown. Although it is very difficult to quantitatively compare PIs between different experiments due to varying PBS backgrounds it seems clear that all constructs more or less are capable of eliciting a proliferative response.

Among the second group of donors some individuals were vaccinated against TT 1-2 months before the experiments. The PIs obtained from these persons (HB, MR, KG, and MW) were all among the highest PIs observed for all the modified TNF α constructs and the data were easy to evaluate. Three other individuals (DL, ID, and LS) were vaccinated more than 5 years back, and they all showed PI values below average. This supports that the observed PIs were antigen specific. No data regarding the last TT vaccination dates were available for the first group of donors, but their immunization status was clearly very variable.

It is not possible to select a preferred TNF α 2 or TNF α 30 protein merely based on the average size of the PI values. This may be due to the heterogeneous vaccination status of the individual used and the variable nature of PBMC assays obtained from individuals with variable responder status. It was anyway surprising, that P2 and P30 at all the inserted positions in TNF α seemed to be able to be processed and presented to T cells. In order to exclude the possibility that despite repeated affinity chromatography, gelfiltration and dialysis, the antigen preparations could still contain significant concentrations of non-specific mitogens, some further experiments were performed.

Donors from the second group known to be non-responders to P2 and responders to P30 as well as donors with the opposite response pattern were tested in PBMC assays. In Fig. 13 the response to P2, P30 as well as the TNF α 2 and TNF α 30 proteins are shown for DL and ID.

It can be seen that specific responses are obtained to the respective T cell epitopes and the respectively modified TNF α molecules. However, no significant proliferative responses of DL against TNF α 30 proteins were observed (upper panel) and no significant proliferative responses of ID to TNF α 2 proteins were observed (lower panel) supporting that non-specific mitogens were absent in the purified TNF α preparations.

This possibility was even further examined by the use of P2 and P30 specific T cell lines isolated from the second group of donors, which had been cultured for at least six weeks in at least three rounds of stimulation with the respective synthetic P2 and P30 peptides. In Fig. 14 the results of two such experiments are shown.

It can be seen that the P2 and P30 specific T cell lines were only stimulated by their corresponding P2 and P30 proteins. Furthermore, it can be seen again that all TNF α constructs are able to induce T cell proliferation emphasizing that although antigen processing may be quantitatively important for presentation of P2 and P30, it does not seem to be a significant qualitative limiting factor for antigen presentation.

It has been reported in the literature that flanking regions of T cell epitopes can influence the binding of antigenic peptides to MHC class II molecules. Since none of the positions in TNF α , which were chosen for insertion of P2 or P30, seemed to be prohibitive for antigen presentation, it was investigated whether the different flanking

TNF α sequences of the inserted epitopes could influence the T cell response to P2 and P30 epitopes as a result of differential binding to the human HLA class II molecules. The peptides shown in Table III, which represent the inserted epitopes as well as the flanking human TNF α amino acids were therefore synthesized. These are designated PP2-5, PP30-3, etc. The amino acid sequences are shown in the sequence listing as SEQ ID NO:34 to SEQ ID NO:42 designated Pep2-1 to Pep30-5.

Table III.

Synthetic peptides representing P2 and P30 and their flanking TNF α regions				
2-1	SRTPSQYIKANSKFIGITELQLQWL	30-1	SRTPSFNNFTVSFWLRVPKVSASHLERRANA	
2-3	SQVLFQYIKANSKFIGITELISRIA	30-2	ALLANFNNFTVSFWLRVPKVSASHLEQVLFK	
2-4	AEAKPQYIKANSKFIGITELGDRLS	30-3	YSQVLFNNFTVSFWLRVPKVSASHLEVSQYT	
2-5	EKGDRQYIKANSKFIGITELSGQVY	30-4	QRETPFNNFTVSFWLRVPKVSASHLEKGDRL	
2-7	ND	30-5	EKGDRFNNFTVSFWLRVPKVSASHLEGIIAL	

These peptides were used for stimulation of P2 and P30 specific T cell lines. The results from stimulation of the P30 lines are shown in Fig. 15. It can be seen that the P2 specific T cell lines from MR and KG shows parallel stimulation patterns when stimulated either with the peptide or with the corresponding TNF α 2 protein. It is clear that TNF2-5 is a good potential antigen and these data further supports that the observed T cell proliferations are antigen specific. There was generally no qualitative differences in the stimulation pattern when the P30 specific T cell lines from MR and KG were stimulated either with peptides or proteins (data not shown). The P30 specific T cell line from HC preferably recognized with TNF30-3 and reacted to a minor extent with TNF30-2.

15 CONCLUSION

Ten differently modified human TNF α proteins have been produced and characterized. They were constructed to contain two well known promiscuous T cell epitopes P2 and P30 in order to be potentially immunogenic in at least 85% of the populations.

All proteins could be expressed and purified although TNF2-4 and TNF30-4 at low levels. Mutations at this position in TNF α also seems to interfere with refolding which results in proteins with poor solubility. No biological TNF α activity could be detected in any of the modified TNF α molecules.

Rabbits were immunized with all ten proteins as well as with native TNF α . After 2-3 months of immunization it was possible to detect high titers of strongly cross-reactive antibodies towards human, non-modified TNF α in all sera.

The ability of these antibodies to interfere with the biological activity of native TNF α was tested in two dif-

ferent *in vitro* assays - the L929 bioassay and a solid phase receptor binding assay. Both assays showed essentially the same TNF2-1, TNF2-4, TNF30-1 and TNF30-4 was not able to induce significant neutralizing antibodies, 5 whereas TNF2-5 was superior compared to the other constructs. TNF30-2 and TNF30-3 were equally efficient at inducing neutralizing antibodies and twice as good as TNF30-5 which was reasonably good.

Somewhat surprisingly it was not possible to see significant differences between the abilities of the molecules 10 to stimulate PBMC to proliferate. It could be demonstrated that this was not due to the presence of mitogens in the antigen preparations and based on these data, it was concluded that the locations chosen for P2 and P30 15 all allowed presentation of the respective epitopes. The specificity of the responses was further documented by using testing the modified TNF α proteins using epitope specific T cell lines as well as synthetic peptides representing the inserted epitopes as well as the flanking 20 TNF α sequences. From these experiments it was clearly demonstrated that TNF2-5, TNF30-2 and TNF30-3 (among other constructs) were the most powerful potential immunogens.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: Farmaceutisk Laboratorium Ferring A/S
- (B) STREET: Indertoften 10
- (C) CITY: Vanløse
- (E) COUNTRY: Denmark
- (F) POSTAL CODE (ZIP): DK-2720

- (ii) TITLE OF INVENTION: Modified human TNF-alpha molecules, DNA encoding them, and vaccines containing said modified TNF-alpha or DNA

- (iii) NUMBER OF SEQUENCES: 42

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 477 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

- (iii) HYPOTHETICAL: NO

- (iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION:1..477
- (C) IDENTIFICATION METHOD: experimental
- (D) OTHER INFORMATION:/codon_start= 1
/function= "Antigen"
/product= "TNF-alpha analog"
/evidence= EXPERIMENTAL
/gene= "tnfP2-1"
/standard_name= "TNF2-1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATG GTC AGA TCA TCT TCT CGA ACC CCG AGT CAG TAC ATT AAA GCC AAT	48
Met Val Arg Ser Ser Ser Arg Thr Pro Ser Gln Tyr Ile Lys Ala Asn	
1 5 10 15	
TCT AAA TTC ATC GGT ATA ACT GAG CTG CAG CTC CAG TGG CTG AAC CGC	96
Ser Lys Phe Ile Gly Ile Thr Glu Leu Gln Leu Gln Trp Leu Asn Arg	
20 25 30	

CGG	GCC	AAT	GCC	CTC	CTG	GCC	AAT	GGC	GTG	GAG	CTG	AGA	GAT	AAC	CAG	144
Arg	Ala	Asn	Ala	Leu	Leu	Ala	Asn	Gly	Val	Glu	Leu	Arg	Asp	Asn	Gln	
		35					40					45				
CTG	GTG	GTG	CCA	TCA	GAG	GGC	CTG	TAC	CTC	ATC	TAC	TCC	CAG	GTC	CTC	192
Leu	Val	Val	Pro	Ser	Glu	Gly	Leu	Tyr	Leu	Ile	Tyr	Ser	Gln	Val	Leu	
	50					55					60					
TTC	AAG	GGC	CAA	GGC	TGC	CCC	TCC	ACC	CAT	GTG	CTC	CTC	ACC	CAC	ACC	240
Phe	Lys	Gly	Gln	Gly	Cys	Pro	Ser	Thr	His	Val	Leu	Leu	Thr	His	Thr	
65					70					75					80	
ATC	AGC	CGC	ATC	GCC	GTC	TCC	TAC	CAG	ACC	AAG	GTC	AAC	CTC	CTC	TCT	288
Ile	Ser	Arg	Ile	Ala	Val	Ser	Tyr	Gln	Thr	Lys	Val	Asn	Leu	Leu	Ser	
				85				90						95		
GCC	ATC	AAG	AGC	CCC	TGC	CAG	AGG	GAG	ACC	CCA	GAG	GGG	GCT	GAG	GCC	336
Ala	Ile	Lys	Ser	Pro	Cys	Gln	Arg	Glu	Thr	Pro	Glu	Gly	Ala	Glu	Ala	
			100					105					110			
AAG	CCC	TGG	TAT	GAG	CCC	ATC	TAT	CTG	GGA	GGG	GTC	TTC	CAG	CTG	GAG	384
Lys	Pro	Trp	Tyr	Glu	Pro	Ile	Tyr	Leu	Gly	Gly	Val	Phe	Gln	Leu	Glu	
		115					120					125				
AAG	GGT	GAC	CGA	CTC	AGC	GCT	GAG	ATC	AAT	CGG	CCC	GAC	TAT	CTC	GAC	432
Lys	Gly	Asp	Arg	Leu	Ser	Ala	Glu	Ile	Asn	Arg	Pro	Asp	Tyr	Leu	Asp	
	130					135					140					
TTT	GCC	GAG	TCT	GGG	CAG	GTC	TAC	TTT	GGG	ATC	ATT	GCC	CTC	TAG		477
Phe	Ala	Glu	Ser	Gly	Gln	Val	Tyr	Phe	Gly	Ile	Ile	Ala	Leu	*		
145					150					155						

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 159 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met	Val	Arg	Ser	Ser	Ser	Arg	Thr	Pro	Ser	Gln	Tyr	Ile	Lys	Ala	Asn	
1				5					10					15		
Ser	Lys	Phe	Ile	Gly	Ile	Thr	Glu	Leu	Gln	Leu	Gln	Trp	Leu	Asn	Arg	
		20						25					30			
Arg	Ala	Asn	Ala	Leu	Leu	Ala	Asn	Gly	Val	Glu	Leu	Arg	Asp	Asn	Gln	
		35					40					45				
Leu	Val	Val	Pro	Ser	Glu	Gly	Leu	Tyr	Leu	Ile	Tyr	Ser	Gln	Val	Leu	
	50					55					60					
Phe	Lys	Gly	Gln	Gly	Cys	Pro	Ser	Thr	His	Val	Leu	Leu	Thr	His	Thr	
65					70					75					80	
Ile	Ser	Arg	Ile	Ala	Val	Ser	Tyr	Gln	Thr	Lys	Val	Asn	Leu	Leu	Ser	
				85					90					95		

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Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala
      100                      105                      110
Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu
      115                      120                      125
Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
      130                      135                      140
Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu *
145                      150                      155

```

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 477 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION:1..477
- (D) OTHER INFORMATION:/codon_start= 1
/function= "Antigen"
/product= "TNF-alpha analog"
/gene= "tnfP2-3"
/standard_name= "TNF2-3"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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ATG GTC AGA TCA TCT TCT CGA ACC CCG AGT GAC AAG CCT GTA GCC CAT      43
Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
160                      165                      170                      175

GTT GTA GCA AAC CCT CAA GCT GAG GGG CAG CTC CAG TGG CTG AAC CGC      95
Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg
180                      185                      190

CGG GCC AAT GCC CTC CTG GCC AAT GGC GTG GAG CTG AGA GAT AAC CAG      144
Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
195                      200                      205

CTG GTG GTG CCA TCA GAG GGC CTG TAC CTC ATC TAC TCC CAG GTC CTC      192
Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
210                      215                      220

TTC CAG TAC ATA AAG GCC AAC TCC AAG TTT ATC GGC ATC ACC GAG CTC      240
Phe Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu
225                      230                      235

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ATC AGC CGC ATC GCC GTC TCC TAC CAG ACC AAG GTC AAC CTC CTC TCT	288
Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser	
240 245 250 255	
GCC ATC AAG AGC CCC TGC CAG AGG GAG ACC CCA GAG GGG GCT GAG GCC	336
Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala	
260 265 270	
AAG CCC TGG TAT GAG CCC ATC TAT CTG GGA GGG GTC TTC CAG CTG GAG	384
Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu	
275 280 285	
AAG GGT GAC CGA CTC AGC GCT GAG ATC AAT CGG CCC GAC TAT CTC GAC	432
Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp	
290 295 300	
TTT GCC GAG TCT GGG CAG GTC TAC TTT GGG ATC ATT GCC CTC TAG	477
Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu *	
305 310 315	

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 159 amino acids

(B) TYPE: amino acid

TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His	
1 5 10 15	
Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg	
20 25 30	
Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln	
35 40 45	
Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu	
50 55 60	
Phe Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu	
65 70 75 80	
Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser	
85 90 95	
Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala	
100 105 110	
Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu	
115 120 125	
Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp	
130 135 140	
Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu *	
145 150 155	

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 477 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

(ix) FEATURE:

- (A) NAME/KEY: CDS
(B) LOCATION:1..477
(D) OTHER INFORMATION:/codon_start= 1
/function= "Antigen"
/product= "TNF-alpha analog"
/gene= "tnfp2-4"
/standard name= "TNF2-4"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

ATG Met 160	GTC Val	AGA Arg	TCA Ser	TCT Ser	TCT Ser	CGA Arg	ACC Thr	CCG Pro	AGT Ser	GAC Asp 170	AAG Lys	CCT Pro	GTA Val	GCC Ala	CAT His 175	48
GTT Val	GTA Val	GCA Ala	AAC Asn	CCT Pro 180	CAA Gln	GCT Ala	GAG Glu	GGG Gly	CAG Gln 185	CTC Leu	CAG Gln	TGG Trp	CTG Leu	AAC Asn 190	CGC Arg	96
CGG Arg	GCC Ala	AAT Asn	GCC Ala 195	CTC Leu	CTG Leu	GCC Ala	AAT Asn	GGC Gly 200	GTG Val	GAG Glu	CTG Leu	AGA Arg	GAT Asp 205	AAC Asn	CAG Gln	144
CTG Leu	GTG Val	GTG Val 210	CCA Pro	TCA Ser	GAG Glu	GGC Gly	CTG Leu 215	TAC Tyr	CTC Leu	ATC Ile	TAC Tyr	TCC Ser 220	CAG Gln	GTC Val	CTC Leu	192
TTC Phe	AAG Lys 225	GGC Gly	CAA Gln	GGC Gly	TGC Cys	CCC Pro 230	TCC Ser	ACC Thr	CAT His	GTG Val	CTC Leu 235	CTC Leu	ACC Thr	CAC His	ACC Thr	240
ATC Ile 240	AGC Ser	CGC Arg	ATC Ile	GCC Ala	GTC Val 245	TCC Ser	TAC Tyr	CAG Gln	ACC Thr	AAG Lys 250	GTC Val	AAC Asn	CTC Leu	CTC Leu	TCT Ser 255	288
GCC Ala	ATC Ile	AAG Lys	AGC Ser	CCC Pro 260	TGC Cys	CAG Gln	AGG Arg	GAG Glu	ACC Thr 265	CCA Pro	GAG Glu	GGG Gly	GCT Ala	GAG Glu 270	GCC Ala	336
AAG Lys	CCC Pro	CAG Gln	TAT Tyr 275	ATC Ile	AAG Lys	GCC Ala	AAT Asn	TCG Ser 280	AAA Lys	TTC Phe	ATC Ile	GGC Gly	ATC Ile 285	ACG Thr	GAG Glu	384
CTC Leu	GGT Gly 290	GAC Asp	CGA Arg	CTC Leu	AGC Ser	GCT Ala	GAG Glu 295	ATC Ile	AAT Asn	CGG Arg	CCC Pro	GAC Asp 300	TAT Tyr	CTC Leu	GAC Asp	432

TTT GCC GAG TCT GGG CAG GTC TAC TTT GGG ATC ATT GCC CTC TAG
 Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu *
 305 310 315

477

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 159 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
 1 5 10 15
 Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg
 20 25 30
 Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
 35 40 45
 Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
 50 55 60
 Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr
 65 70 75 80
 Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
 85 90 95
 Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala
 100 105 110
 Lys Pro Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu
 115 120 125
 Leu Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
 130 135 140
 Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu *
 145 150 155

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 477 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..477

(D) OTHER INFORMATION: /function= "Antigen"

/product= "TNF-alpha analog"

/gene= "tnfP2-5"

/standard_name= "TNF2-5"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

ATG GTC AGA TCA TCT TCT CGA ACC CCG AGT GAC AAG CCT GTA GCC CAT	48
Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His	
160 165 170 175	
GTT GTA GCA AAC CCT CAA GCT GAG GGG CAG CTC CAG TGG CTG AAC CGC	96
Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg	
180 185 190	
CGG GCC AAT GCC CTC CTG GCC AAT GGC GTG GAG CTG AGA GAT AAC CAG	144
Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln	
195 200 205	
CTG GTG GTG CCA TCA GAG GGC CTG TAC CTC ATC TAC TCC CAG GTC CTC	192
Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu	
210 215 220	
TTC AAG GGC CAA GGC TGC CCC TCC ACC CAT GTG CTC CTC ACC CAC ACC	240
Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr	
225 230 235	
ATC AGC CGC ATC GCC GTC TCC TAC CAG ACC AAG GTC AAC CTC CTC TCT	288
Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser	
240 245 250 255	
GCC ATC AAG AGC CCC TGC CAG AGG GAG ACC CCA GAG GGG GCT GAG GCC	336
Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala	
260 265 270	
AAG CCC TGG TAT GAG CCC ATC TAT CTG GGA GGG GTC TTC CAG CTG GAG	384
Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu	
275 280 285	
AAG GGT GAC CGA CAG TAC ATT AAG GCC AAT TCG AAG TTC ATT GGC ATC	432
Lys Gly Asp Arg Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile	
290 295 300	
ACT GAG CTG TCT GGG CAG GTC TAC TTT GGG ATC ATT GCC CTC TAG	477
Thr Glu Leu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu *	
305 310 315	

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 159 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

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Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
 1           5           10           15
Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg
           20           25           30
Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
           35           40           45
Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
 50           55           60
Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr
 65           70           75           80
Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
           85           90           95
Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala
           100          105          110
Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu
           115          120          125
Lys Gly Asp Arg Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile
 130          135          140
Thr Glu Leu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu *
145          150          155

```

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 477 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..477
- (D) OTHER INFORMATION: /codon_start= 1
 - /function= "Antigen"
 - /product= "TNF-alpha analog"
 - /gene= "tnfp2-7"
 - /standard_name= "TNF2-7"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

ATG	GTC	AGA	TCA	TCT	TCT	CGA	ACC	CCG	AGT	GAC	AAG	CCT	GTA	GCC	CAT	43
Met	Val	Arg	Ser	Ser	Ser	Arg	Thr	Pro	Ser	Asp	Lys	Pro	Val	Ala	His	
160						165				170					175	
GTT	GTA	GCA	AAC	CCT	CAA	GCT	GAG	GGG	CAG	CTC	CAG	TGG	CTG	AAC	CGC	86
Val	Val	Ala	Asn	Pro	Gln	Ala	Glu	Gly	Gln	Leu	Gln	Trp	Leu	Asn	Arg	
				180					185						190	
CGG	GCC	AAT	GCC	CTC	CTG	GCC	AAT	GGC	GTG	GAG	CTG	AGA	GAT	AAC	CAG	144
Arg	Ala	Asn	Ala	Leu	Leu	Ala	Asn	Gly	Val	Glu	Leu	Arg	Asp	Asn	Gln	
			195					200					205			
CTG	GTG	GTG	CCA	TCA	GAG	GGC	CTG	TAC	CTC	ATC	TAC	TCC	CAG	GTC	CTC	192
Leu	Val	Val	Pro	Ser	Glu	Gly	Leu	Tyr	Leu	Ile	Tyr	Ser	Gln	Val	Leu	
		210					215					220				
TTC	AAG	GGC	CAA	GGC	TGC	CCC	TCC	ACC	CAT	GTG	CTC	CAG	TAC	ATC	AAA	240
Phe	Lys	Gly	Gln	Gly	Cys	Pro	Ser	Thr	His	Val	Leu	Gln	Tyr	Ile	Lys	
	225					230					235					
GCT	AAC	TCC	AAA	TTC	ATC	GGC	ATC	ACC	GAA	CTG	GTT	AAC	CTC	CTC	TCT	288
Ala	Asn	Ser	Lys	Phe	Ile	Gly	Ile	Thr	Glu	Leu	Val	Asn	Leu	Leu	Ser	
240					245					250					255	
GCC	ATC	AAG	AGC	CCC	TGC	CAG	AGG	GAG	ACC	CCA	GAG	GGG	GCT	GAG	GCC	336
Ala	Ile	Lys	Ser	Pro	Cys	Gln	Arg	Glu	Thr	Pro	Glu	Gly	Ala	Glu	Ala	
				260					265						270	
AAG	CCC	TGG	TAT	GAG	CCC	ATC	TAT	CTG	GGA	GGG	GTC	TTC	CAG	CTG	GAG	384
Lys	Pro	Trp	Tyr	Glu	Pro	Ile	Tyr	Leu	Gly	Gly	Val	Phe	Gln	Leu	Glu	
			275					280					285			
AAG	GGT	GAC	CGA	CTC	AGC	GCT	GAG	ATC	AAT	CGG	CCC	GAC	TAT	CTC	GAC	432
Lys	Gly	Asp	Arg	Leu	Ser	Ala	Glu	Ile	Asn	Arg	Pro	Asp	Tyr	Leu	Asp	
		290					295					300				
TTT	GCC	GAG	TCT	GGG	CAG	GTC	TAC	TTT	GGG	ATC	ATT	GCC	CTC	TAG		477
Phe	Ala	Glu	Ser	Gly	Gln	Val	Tyr	Phe	Gly	Ile	Ile	Ala	Leu	*		
	305					310				315						

(2) INFORMATION FOR SEQ ID NO: 10:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 159 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Met	Val	Arg	Ser	Ser	Ser	Arg	Thr	Pro	Ser	Asp	Lys	Pro	Val	Ala	His
1				5					10					15	
Val	Val	Ala	Asn	Pro	Gln	Ala	Glu	Gly	Gln	Leu	Gln	Trp	Leu	Asn	Arg
			20					25					30		
Arg	Ala	Asn	Ala	Leu	Leu	Ala	Asn	Gly	Val	Glu	Leu	Arg	Asp	Asn	Gln
		35					40					45			

```

Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
 50                      55                      60
Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Gln Tyr Ile Lys
 65                      70                      75                      80
Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu Val Asn Leu Leu Ser
                      85                      90                      95
Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala
      100                      105                      110
Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu
      115                      120                      125
Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
 130                      135                      140
Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu *
 145                      150                      155

```

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 477 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..477
- (D) OTHER INFORMATION: /codon_start= 1
 - /function= "Antigen"
 - /product= "TNF-alpha analog"
 - /gene= "tnfP30-1"
 - /standard_name= "TNF30-1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

```

ATG GTC AGA TCA TCT TCT CGA ACC CCG AGT TTC AAC AAT TTT ACC GTA      48
Met Val Arg Ser Ser Arg Thr Pro Ser Phe Asn Asn Phe Thr Val
 160                      165                      170                      175

AGC TTT TGG CTC CGT GTA CCT AAG GTG TCG GCC TCG CAC CTG GAG CGC      96
Ser Phe Trp Leu Arg Val Pro Lys Val Ser Ala Ser His Leu Glu Arg
      180                      185                      190

CGG GCC AAT GCC CTC CTG GCC AAT GGC GTG GAG CTG AGA GAT AAC CAG      144
Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
      195                      200                      205

```

CTG	GTG	GTG	CCA	TCA	GAG	GGC	CTG	TAC	CTC	ATC	TAC	TCC	CAG	GTC	CTC	192
Leu	Val	Val	Pro	Ser	Glu	Gly	Leu	Tyr	Leu	Ile	Tyr	Ser	Gln	Val	Leu	
		210					215					220				
TTC	AAG	GGC	CAA	GGC	TGC	CCC	TCC	ACC	CAT	GTG	CTC	CTC	ACC	CAC	ACC	240
Phe	Lys	Gly	Gln	Gly	Cys	Pro	Ser	Thr	His	Val	Leu	Leu	Thr	His	Thr	
	225					230					235					
ATC	AGC	CGC	ATC	GCC	GTC	TCC	TAC	CAG	ACC	AAG	GTC	AAC	CTC	CTC	TCT	288
Ile	Ser	Arg	Ile	Ala	Val	Ser	Tyr	Gln	Thr	Lys	Val	Asn	Leu	Leu	Ser	
240					245					250					255	
GCC	ATC	AAG	AGC	CCC	TGC	CAG	AGG	GAG	ACC	CCA	GAG	GGG	GCT	GAG	GCC	336
Ala	Ile	Lys	Ser	Pro	Cys	Gln	Arg	Glu	Thr	Pro	Glu	Gly	Ala	Glu	Ala	
				260					265					270		
AAG	CCC	TGG	TAT	GAG	CCC	ATC	TAT	CTG	GGA	GGG	GTC	TTC	CAG	CTG	GAG	384
Lys	Pro	Trp	Tyr	Glu	Pro	Ile	Tyr	Leu	Gly	Gly	Val	Phe	Gln	Leu	Glu	
		275						280					285			
AAG	GGT	GAC	CGA	CTC	AGC	GCT	GAG	ATC	AAT	CGG	CCC	GAC	TAT	CTC	GAC	432
Lys	Gly	Asp	Arg	Leu	Ser	Ala	Glu	Ile	Asn	Arg	Pro	Asp	Tyr	Leu	Asp	
		290					295					300				
TTT	GCC	GAG	TCT	GGG	CAG	GTC	TAC	TTT	GGG	ATC	ATT	GCC	CTC	TAG		477
Phe	Ala	Glu	Ser	Gly	Gln	Val	Tyr	Phe	Gly	Ile	Ile	Ala	Leu	*		
	305					310					315					

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 159 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Met	Val	Arg	Ser	Ser	Ser	Arg	Thr	Pro	Ser	Phe	Asn	Asn	Phe	Thr	Val	
1				5				10						15		
Ser	Phe	Trp	Leu	Arg	Val	Pro	Lys	Val	Ser	Ala	Ser	His	Leu	Glu	Arg	
		20					25						30			
Arg	Ala	Asn	Ala	Leu	Leu	Ala	Asn	Gly	Val	Glu	Leu	Arg	Asp	Asn	Gln	
		35					40					45				
Leu	Val	Val	Pro	Ser	Glu	Gly	Leu	Tyr	Leu	Ile	Tyr	Ser	Gln	Val	Leu	
	50					55					60					
Phe	Lys	Gly	Gln	Gly	Cys	Pro	Ser	Thr	His	Val	Leu	Leu	Thr	His	Thr	
65				70						75				80		
Ile	Ser	Arg	Ile	Ala	Val	Ser	Tyr	Gln	Thr	Lys	Val	Asn	Leu	Leu	Ser	
			85					90					95			
Ala	Ile	Lys	Ser	Pro	Cys	Gln	Arg	Glu	Thr	Pro	Glu	Gly	Ala	Glu	Ala	
			100					105					110			
Lys	Pro	Trp	Tyr	Glu	Pro	Ile	Tyr	Leu	Gly	Gly	Val	Phe	Gln	Leu	Glu	
	115						120					125				

Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
 130 135 140
 Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu *
 145 150 155

(2) INFORMATION FOR SEQ ID NO: 13:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 477 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
 (A) ORGANISM: Homo sapiens

(ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION:1..477
 (D) OTHER INFORMATION:/codon_start= 1
 /function= "Antigen"
 /product= "TNF-alpha analog"
 /gene= "tnfP30-2"
 /standard_name= "TNF30-2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

ATG GTC AGA TCA TCT TCT CGA ACC CCG AGT GAC AAG CCT GTA GCC CAT	48
Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His	
160 165 170 175	
GTT GTA GCA AAC CCT CAA GCT GAG GGG CAG CTC CAG TGG CTG AAC CGC	96
Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg	
180 185 190	
CGG GCC AAT GCC CTC CTG GCC AAT TTC AAC AAC TTC ACA GTT AGC TTC	144
Arg Ala Asn Ala Leu Leu Ala Asn Phe Asn Asn Phe Thr Val Ser Phe	
195 200 205	
TGG TTG AGG GTA CCA AAG GTC TCG GCC AGC CAC CTC GAG CAG GTC CTC	192
Trp Leu Arg Val Pro Lys Val Ser Ala Ser His Leu Glu Gln Val Leu	
210 215 220	
TTC AAG GGC CAA GGC TGC CCC TCC ACC CAT GTG CTC CTC ACC CAC ACC	240
Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr	
225 230 235	
ATC AGC CGC ATC GCC GTC TCC TAC CAG ACC AAG GTC AAC CTC CTC TCT	288
Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser	
240 245 250 255	
GCC ATC AAG AGC CCC TGC CAG AGG GAG ACC CCA GAG GGG GCT GAG GCC	336
Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala	
260 265 270	

AAG CCC TGG TAT GAG CCC ATC TAT CTG GGA GGG GTC TTC CAG CTG GAG	384
Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu	
275 280 285	
AAG GGT GAC CGA CTC AGC GCT GAG ATC AAT CGG CCC GAC TAT CTC GAC	432
Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp	
290 295 300	
TTT GCC GAG TCT GGG CAG GTC TAC TTT GGG ATC ATT GCC CTC TAG	477
Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu *	
305 310 315	

(2) INFORMATION FOR SEQ ID NO: 14:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 159 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His	
1 5 10 15	
Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg	
20 25 30	
Arg Ala Asn Ala Leu Leu Ala Asn Phe Asn Asn Phe Thr Val Ser Phe	
35 40 45	
Trp Leu Arg Val Pro Lys Val Ser Ala Ser His Leu Glu Gln Val Leu	
50 55 60	
Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr	
65 70 75 80	
Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser	
85 90 95	
Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala	
100 105 110	
Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu	
115 120 125	
Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp	
130 135 140	
Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu *	
145 150 155	

(2) INFORMATION FOR SEQ ID NO: 15:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 477 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION:1..477

(D) OTHER INFORMATION:/codon_start= 1

/function= "Antigen"

/product= "TNF-alpha analog"

/gene= "tnfp30-3"

/standard_name= "TNF30-3"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

ATG GTC AGA TCA TCT TCT CGA ACC CCG AGT GAC AAG CCT GTA GCC CAT	48
Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His	
160 165 170 175	
GTT GTA GCA AAC CCT CAA GCT GAG GGG CAG CTC CAG TGG CTG AAC CGC	96
Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg	
180 185 190	
CGG GCC AAT GCC CTC CTG GCC AAT GGC GTG GAG CTG AGA GAT AAC CAG	144
Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln	
195 200 205	
CTG GTG GTG CCA TCA GAG GGC CTG TAC CTC ATC TAC TCC CAG GTC CTC	192
Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu	
210 215 220	
TTC AAC AAC TTT ACC GTC TCC TTC TGG CTT CGG GTA CCC AAG GTC AGC	240
Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser	
225 230 235	
GCT AGC CAC CTC GAG GTC TCC TAC CAG ACC AAG GTC AAC CTC CTC TCT	288
Ala Ser His Leu Glu Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser	
240 245 250 255	
GCC ATC AAG AGC CCC TGC CAG AGG GAG ACC CCA GAG GGG GCT GAG GCC	336
Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala	
260 265 270	
AAG CCC TGG TAT GAG CCC ATC TAT CTG GGA GGG GTC TTC CAG CTG GAG	384
Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu	
275 280 285	
AAG GGT GAC CGA CTC AGC GCT GAG ATC AAT CGG CCC GAC TAT CTC GAC	432
Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp	
290 295 300	
TTT GCC GAG TCT GGG CAG GTC TAC TTT GGG ATC ATT GCC CTC TAG	477
Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu *	
305 310 315	

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 159 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

```

Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
 1           5           10           15
Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg
          20           25           30
Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
          35           40           45
Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
 50           55           60
Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser
 65           70           75           80
Ala Ser His Leu Glu Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
          85           90           95
Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala
          100          105          110
Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu
          115          120          125
Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
          130          135          140
Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu *
145          150          155

```

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 477 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..477

(D) OTHER INFORMATION:/function= "Antigen"
 /product= "TNF-alpha analog"
 /gene= "tnfP30-4"
 /standard_name= "TNF30-4"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

ATG GTC AGA TCA TCT TCT CGA ACC CCG AGT GAC AAG CCT GTA GCC CAT	48
Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His	
160 165 170 175	
GTT GTA GCA AAC CCT CAA GCT GAG GGG CAG CTC CAG TGG CTG AAC CGC	96
Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg	
180 185 190	
CGG GCC AAT GCC CTC CTG GCC AAT GGC GTG GAG CTG AGA GAT AAC CAG	144
Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln	
195 200 205	
CTG GTG GTG CCA TCA GAG GGC CTG TAC CTC ATC TAC TCC CAG GTC CTC	192
Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu	
210 215 220	
TTC AAG GGC CAA GGC TGC CCC TCC ACC CAT GTG CTC CTC ACC CAC ACC	240
Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr	
225 230 235	
ATC AGC CGC ATC GCC GTC TCC TAC CAG ACC AAG GTC AAC CTC CTC TCT	288
Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser	
240 245 250 255	
GCC ATC AAG AGC CCC TGC CAG AGG GAG ACC CCA TTT AAT AAT TTC ACC	336
Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Phe Asn Asn Phe Thr	
260 265 270	
GTG TCC TTC TGG TTG CGC GTC CCT AAG GTA AGC GCT TCC CAC CTG GAG	384
Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser Ala Ser His Leu Glu	
275 280 285	
AAG GGT GAC CGA CTC AGC GCT GAG ATC AAT CGG CCC GAC TAT CTC GAC	432
Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp	
290 295 300	
TTT GCC GAG TCT GGG CAG GTC TAC TTT GGG ATC ATT GCC CTC TAG	477
Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu *	
305 310 315	

(2) INFORMATION FOR SEQ ID NO: 18:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 159 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
1 5 10 15

```

Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg
      20      25      30
Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
      35      40      45
Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
      50      55      60
Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr
      65      70      75      80
Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
      85      90      95
Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Phe Asn Asn Phe Thr
      100      105      110
Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser Ala Ser His Leu Glu
      115      120      125
Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
      130      135      140
Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu *
      145      150      155

```

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 477 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..477
- (D) OTHER INFORMATION: /codon_start= 1
/function= "Antigen"
/product= "TNF-alpha analog"
/gene= "tnfp30-5"
/standard_name= "TNF30-5"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

```

ATG GTC AGA TCA TCT TCT CGA ACC CCG AGT GAC AAG CCT GTA GCC CAT      48
Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
160      165      170      175

GTT GTA GCA AAC CCT CAA GCT GAG GGG CAG CTC CAG TGG CTG AAC CGC      96
Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg
      180      185      190

```

CGG	GCC	AAT	GCC	CTC	CTG	GCC	AAT	GGC	GTG	GAG	CTG	AGA	GAT	AAC	CAG	144
Arg	Ala	Asn	Ala	Leu	Leu	Ala	Asn	Gly	Val	Glu	Leu	Arg	Asp	Asn	Gln	
			195					200					205			
CTG	GTG	GTG	CCA	TCA	GAG	GGC	CTG	TAC	CTC	ATC	TAC	TCC	CAG	GTC	CTC	192
Leu	Val	Val	Pro	Ser	Glu	Gly	Leu	Tyr	Leu	Ile	Tyr	Ser	Gln	Val	Leu	
		210					215					220				
TTC	AAG	GGC	CAA	GGC	TGC	CCC	TCC	ACC	CAT	GTG	CTC	CTC	ACC	CAC	ACC	240
Phe	Lys	Gly	Gln	Gly	Cys	Pro	Ser	Thr	His	Val	Leu	Leu	Thr	His	Thr	
	225					230					235					
ATC	AGC	CGC	ATC	GCC	GTC	TCC	TAC	CAG	ACC	AAG	GTC	AAC	CTC	CTC	TCT	288
Ile	Ser	Arg	Ile	Ala	Val	Ser	Tyr	Gln	Thr	Lys	Val	Asn	Leu	Leu	Ser	
240					245					250					255	
GCC	ATC	AAG	AGC	CCC	TGC	CAG	AGG	GAG	ACC	CCA	GAG	GGG	GCT	GAG	GCC	336
Ala	Ile	Lys	Ser	Pro	Cys	Gln	Arg	Glu	Thr	Pro	Glu	Gly	Ala	Glu	Ala	
			260					265					270			
AAG	CCC	TGG	TAT	GAG	CCC	ATC	TAT	CTG	GGA	GGG	GTC	TTC	CAG	CTG	GAG	384
Lys	Pro	Trp	Tyr	Glu	Pro	Ile	Tyr	Leu	Gly	Gly	Val	Phe	Gln	Leu	Glu	
		275					280						285			
AAG	GGT	GAC	CGA	TTC	AAC	AAT	TTC	ACC	GTA	AGC	TTC	TGG	CTT	CGC	GTC	432
Lys	Gly	Asp	Arg	Phe	Asn	Asn	Phe	Thr	Val	Ser	Phe	Trp	Leu	Arg	Val	
		290				295						300				
CCT	AAG	GTG	TCT	GCG	TCG	CAC	CTC	GAA	GGG	ATC	ATT	GCC	CTC	TAG		477
Pro	Lys	Val	Ser	Ala	Ser	His	Leu	Glu	Gly	Ile	Ile	Ala	Leu	*		
	305					310					315					

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 159 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

Met	Val	Arg	Ser	Ser	Ser	Arg	Thr	Pro	Ser	Asp	Lys	Pro	Val	Ala	His	
1				5					10					15		
Val	Val	Ala	Asn	Pro	Gln	Ala	Glu	Gly	Gln	Leu	Gln	Trp	Leu	Asn	Arg	
		20					25					30				
Arg	Ala	Asn	Ala	Leu	Leu	Ala	Asn	Gly	Val	Glu	Leu	Arg	Asp	Asn	Gln	
	35					40						45				
Leu	Val	Val	Pro	Ser	Glu	Gly	Leu	Tyr	Leu	Ile	Tyr	Ser	Gln	Val	Leu	
	50					55					60					
Phe	Lys	Gly	Gln	Gly	Cys	Pro	Ser	Thr	His	Val	Leu	Leu	Thr	His	Thr	
65				70					75					80		
Ile	Ser	Arg	Ile	Ala	Val	Ser	Tyr	Gln	Thr	Lys	Val	Asn	Leu	Leu	Ser	
			85					90						95		

Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala
 100 105 110

Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu
 115 120 125

Lys Gly Asp Arg Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg Val
 130 135 140

Pro Lys Val Ser Ala Ser His Leu Glu Gly Ile Ile Ala Leu +
 145 150 155

(2) INFORMATION FOR SEQ ID NO: 21:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (ix) FEATURE:
 - (A) NAME/KEY: misc_feature
 - (B) LOCATION: 1..24
 - (C) IDENTIFICATION METHOD: experimental
 - (D) OTHER INFORMATION: /function= "Primer for PCR cloning
 of DNA encoding TNF-alpha"
 /product= "Primer binding to TNF-alpha gene"
 /evidence= EXPERIMENTAL
 /standard_name= "TNF-alpha Primer I"
 /label= Primer1
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

GACAAGCCCA TGGTCAGATC ATCT

24

(2) INFORMATION FOR SEQ ID NO: 22:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (ix) FEATURE:
 - (A) NAME/KEY: misc_feature
 - (B) LOCATION: 1..30
 - (C) IDENTIFICATION METHOD: experimental

(D) OTHER INFORMATION:/function= "Primer for PCR cloning
of DNA encoding TNF-alpha"
/product= "Primer binding to TNF-alpha gene"
/evidence= EXPERIMENTAL
/standard_name= "TNF-alpha Primer II"
/label= Primer2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

TCTCTAGAGG GCAATGATCC CAAAGTAGAC

30

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

(A) NAME/KEY: misc_feature
(B) LOCATION:1..21
(C) IDENTIFICATION METHOD: experimental
(D) OTHER INFORMATION:/function= "Primer for PCR cloning
of DNA encoding TNF-alpha"
/product= "Primer binding to TNF-alpha gene"
/evidence= EXPERIMENTAL
/standard_name= "TNF-alpha Primer III"
/label= Primer3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

CCCAAAGTAG ACCTGCCCAG A

21

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 69 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(ix) FEATURE:

(A) NAME/KEY: insertion_seq
(B) LOCATION:7..51
(C) IDENTIFICATION METHOD: experimental

(D) OTHER INFORMATION:/function= "Primer for PCR cloning
of DNA encoding TNF-alpha analog"
/evidence= EXPERIMENTAL
/organism= "Homo sapiens"
/standard_name= "Primer "mut2-1"
/label= mut2-1
/note= "Primer "mut2-1" is a synthetically synthesised
69-mer oligonucleotide comprising DNA encoding the human
T cell epitope P2 between stretches of DNA homologous to
stretches of the human TNF-alpha gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

ACCCCGAGTC AGTACATTAA AGCCAATTCT AAATTCATCG GTATAACTGA GCTGCAGCTC 60
CAGTGGCTG 69

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 73 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(ix) FEATURE:

(A) NAME/KEY: insertion_seq
(B) LOCATION:15..59
(C) IDENTIFICATION METHOD: experimental
(D) OTHER INFORMATION:/function= "Primer for PCR cloning
of DNA encoding TNF-alpha analog"
/evidence= EXPERIMENTAL
/organism= "Homo sapiens"
/standard_name= "Primer "mut2-3"
/label= mut2-3
/note= "Primer "mut2-3" is a synthetically synthesised
73-mer oligonucleotide comprising DNA encoding the human
T cell epitope P2 between stretches of DNA homologous to
stretches of the human TNF-alpha gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

CCCAGGTCCT CTTCCAGTAC ATAAAGGCCA ACTCCAAGTT TATCGGCATC ACCGAGCTCA 60
TCAGCCGCAT CGC 73

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 75 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Homo sapiens
- (ix) FEATURE:
 - (A) NAME/KEY: insertion_seq
 - (B) LOCATION:12..56
 - (C) IDENTIFICATION METHOD: experimental
 - (D) OTHER INFORMATION:/function= "Primer for PCR cloning of DNA encoding TNF-alpha analog"
 - /evidence= EXPERIMENTAL
 - /organism= "Homo sapiens"
 - /standard_name= "Primer "mut2-4""
 - /label= mut2-4
 - /note= "Primer "mut2-4" is a synthetically synthesised 75-mer oligonucleotide comprising DNA encoding the human T cell epitope P2 between stretches of DNA homologous to stretches of the human TNF-alpha gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

```
AGTCGGTCAC CGAGCTCCGT GATGCCGATG AATTTCGAAT TGGCCTTGAT ATACTGGGGC      60
TTGGCCTCAG CCCCCC                                                    75
```

(2) INFORMATION FOR SEQ ID NO: 27:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 75 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Homo sapiens
- (ix) FEATURE:
 - (A) NAME/KEY: insertion_seq
 - (B) LOCATION:8..52
 - (C) IDENTIFICATION METHOD: experimental
 - (D) OTHER INFORMATION:/function= "Primer for PCR cloning of DNA encoding TNF-alpha analog"
 - /evidence= EXPERIMENTAL
 - /organism= "Homo sapiens"
 - /standard_name= "Primer "mut2-5""
 - /label= mut2-5
 - /note= "Primer "mut2-5" is a synthetically synthesised 75-mer oligonucleotide comprising DNA encoding the human T cell epitope P2 between stretches of DNA homologous to stretches of the human TNF-alpha gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

GAAGGGTGAC CGACAGTACA TTAAGGCCAA TTCGAAGTTC ATTGGCATCA CTGAGCTGTC 60
TGGGCAGGTC TACTT 75

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 80 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

(ix) FEATURE:

- (A) NAME/KEY: insertion_seq
- (B) LOCATION:14..58
- (C) IDENTIFICATION METHOD: experimental
- (D) OTHER INFORMATION:/function= "Primer for PCR cloning
of DNA encoding TNF-alpha analog"
/evidence= EXPERIMENTAL
/organism= "Homo sapiens"
/standard_name= "Primer "mut2-7"
/label= mut2-7
/note= "Primer "mut2-7" is a synthetically synthesised
80-mer oligonucleotide comprising DNA encoding the human
T cell epitope P2 between stretches of DNA homologous to
stretches of the human TNF-alpha gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

CACCCATGTG CTCCAGTACA TCAAAGCTAA CTCCAAATTC ATCGGCATCA CCGAACTGGT 60
TAACCTCCTC TCTGCCATCA 80

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 96 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

(ix) FEATURE:

- (A) NAME/KEY: insertion_seq
- (B) LOCATION:10..72
- (C) IDENTIFICATION METHOD: experimental
- (D) OTHER INFORMATION:/function= "Primer for PCR cloning of DNA encoding TNF-alpha analog"
 /evidence= EXPERIMENTAL
 /organism= "Homo sapiens"
 /standard_name= "Primer "mut30-1"
 /label= mut30-1
 /note= "Primer "mut30-1" is a synthetically synthesised 96-mer oligonucleotide comprising DNA encoding the human T cell epitope P30 between stretches of DNA homologous to stretches of the human TNF-alpha gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

ACCCCGAGTT TCAACAATTT TACCGTAAGC TTTTGGCTCC GTGTACCTAA GGTGTCGGCC 60
 TCGCACCTGG AGCGCCGGGC CAATGCCCTC CTGGCC 96

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 100 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

(ix) FEATURE:

- (A) NAME/KEY: insertion_seq
- (B) LOCATION:12..74
- (C) IDENTIFICATION METHOD: experimental
- (D) OTHER INFORMATION:/function= "Primer for PCR cloning of DNA encoding TNF-alpha analog"
 /evidence= EXPERIMENTAL
 /organism= "Homo sapiens"
 /standard_name= "Primer "mut30-2"
 /label= mut30-2
 /note= "Primer "mut30-2" is a synthetically synthesised 100-mer oligonucleotide comprising DNA encoding human T cell epitope P30 between stretches of DNA homologous to stretches of the human TNF-alpha gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

TCCTGGCCAA TTTCAACAAC TTCACAGTTA GCTTCTGGTT GAGGGTACCA AAGGTCTCGG 60
 CCAGCCACCT CGAGCAGGTC CTCTTCAAGG GCCAAGGCTG 100

(2) INFORMATION FOR SEQ ID NO: 31:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 100 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Homo sapiens
- (ix) FEATURE:
 - (A) NAME/KEY: insertion_seq
 - (B) LOCATION:12..74
 - (C) IDENTIFICATION METHOD: experimental
 - (D) OTHER INFORMATION:/function= "Primer for PCR cloning of DNA encoding TNF-alpha analog"
 /evidence= EXPERIMENTAL
 /organism= "Homo sapiens"
 /standard_name= "Primer "mut30-3"
 /label= mut30-3
 /note= "Primer "mut30-3" is a synthetically synthesised 100-mer oligonucleotide comprising DNA encoding human T cell epitope P30 between stretches of DNA homologous to stretches of the human TNF-alpha gene"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

```

CCCAGGTCCT CTTCAACAAC TTTACCGTCT CCTTCTGGCT TCGGGTACCC AAGGTCAGCG      60
CTAGCCACCT CGAGGTCTCC TACCAGACCA AGGTCAACCT                               100

```

(2) INFORMATION FOR SEQ ID NO: 32:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 100 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Homo sapiens
- (ix) FEATURE:
 - (A) NAME/KEY: insertion_seq
 - (B) LOCATION:15..77
 - (C) IDENTIFICATION METHOD: experimental

(D) OTHER INFORMATION:/function= "Primer for PCR cloning
of DNA encoding TNF-alpha analog"
/evidence= EXPERIMENTAL
/organism= "Homo sapiens"
/standard name= "Primer "mut30-4""
/label= mut30-4
/note= "Primer "mut30-4" is a synthetically synthesised
100-mer oligonucleotide comprising DNA encoding human T
cell epitope P30 between stretches of DNA homologous to
stretches of the human TNF-alpha gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

```
AGTCGGTCAC CCTTCTCCAG GTGGGAAGCG CTTACCTTAG GGACGCGCAA CCAGAAGGAC      60
ACGGTGAAAT TATTAAATGG GGTCTCCCTC TGGCAGGGGC                          100
```

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 100 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

(ix) FEATURE:

- (A) NAME/KEY: insertion_seq
- (B) LOCATION:14..76
- (C) IDENTIFICATION METHOD: experimental
- (D) OTHER INFORMATION:/function= "Primer for PCR cloning
of DNA encoding TNF-alpha analog"
/evidence= EXPERIMENTAL
/organism= "Homo sapiens"
/standard_name= "Primer "mut30-5""
/label= mut30-5
/note= "Primer "mut30-5" is a synthetically synthesised
100-mer oligonucleotide comprising DNA encoding human T
cell epitope P30 between stretches of DNA homologous to
stretches of the human TNF-alpha gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

```
GAAGGGTGAC CGATTCAACA ATTTACCGT AAGCTTCTGG CTTGCGTCC CTAAGGTGTC      60
TGGTTCGCAC CTCGAAGGGA TCATTGCCCT CTAGAGTCGA                          100
```

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION:1..25
(D) OTHER INFORMATION:/label= Pep2-1
/note= "Pep2-1 is a synthetically prepared truncated form
of a TNF-alpha analog comprising the human T cell epitope
P2 and flanking portions of human TNF-alpha"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

Ser	Arg	Thr	Pro	Ser	Gln	Tyr	Ile	Lys	Ala	Asn	Ser	Lys	Phe	Ile	Gly
1				5				10						15	
Ile	Thr	Glu	Leu	Gln	Leu	Gln	Trp	Leu							
			20				25								

(2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION:1..25
(D) OTHER INFORMATION:/label= Pep2-3
/note= "Pep2-3 is a synthetically prepared truncated form
of a TNF-alpha analog comprising the human T cell epitope
P2 and flanking portions of human TNF-alpha"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

Ser	Gln	Val	Leu	Phe	Gln	Tyr	Ile	Lys	Ala	Asn	Ser	Lys	Phe	Ile	Gly
1				5				10						15	
Ile	Thr	Glu	Leu	Ile	Ser	Arg	Ile	Ala							
			20				25								

(2) INFORMATION FOR SEQ ID NO: 36:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- (v) FRAGMENT TYPE: internal
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Homo sapiens
- (ix) FEATURE:
 - (A) NAME/KEY: Peptide
 - (B) LOCATION:1..25
 - (D) OTHER INFORMATION:/label= Pep2-4
 /note= "Pep2-4 is a synthetically prepared truncated form
 of a TNF-alpha analog comprising the human T cell epitope
 P2 and flanking portions of human TNF-alpha"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

Ala	Glu	Ala	Lys	Pro	Gln	Tyr	Ile	Lys	Ala	Asn	Ser	Lys	Phe	Ile	Gly
1				5				10						15	
Ile	Thr	Glu	Leu	Gly	Asp	Arg	Leu	Ser							
			20				25								

(2) INFORMATION FOR SEQ ID NO: 37:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- (v) FRAGMENT TYPE: internal
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Homo sapiens
- (ix) FEATURE:
 - (A) NAME/KEY: Peptide
 - (B) LOCATION:1..25
 - (D) OTHER INFORMATION:/label= Pep2-5
 /note= "Pep2-5 is a synthetically prepared truncated form
 of a TNF-alpha analog comprising the human T cell epitope
 P2 and flanking portions of human TNF-alpha"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

Glu	Lys	Gly	Asp	Arg	Gln	Tyr	Ile	Lys	Ala	Asn	Ser	Lys	Phe	Ile	Gly
1				5				10						15	

Ile Thr Glu Leu Ser Gly Gln Val Tyr
 20 25

(2) INFORMATION FOR SEQ ID NO: 38:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 31 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- (v) FRAGMENT TYPE: internal
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Homo sapiens
- (ix) FEATURE:
 - (A) NAME/KEY: Peptide
 - (B) LOCATION:1..31
 - (D) OTHER INFORMATION:/label= Pep30-1
 /note= "Pep30-1 is a synthetically prepared truncated
 form of a TNF-alpha analog comprising human T cell
 epitope P30 and flanking portions of human TNF-alpha"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

Ser	Arg	Thr	Pro	Ser	Phe	Asn	Asn	Phe	Thr	Val	Ser	Phe	Trp	Leu	Arg
1				5				10						15	
Val	Pro	Lys	Val	Ser	Ala	Ser	His	Leu	Glu	Arg	Arg	Ala	Asn	Ala	
			20					25					30		

(2) INFORMATION FOR SEQ ID NO: 39:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 31 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- (v) FRAGMENT TYPE: internal
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Homo sapiens
- (ix) FEATURE:
 - (A) NAME/KEY: Peptide
 - (B) LOCATION:1..31
 - (D) OTHER INFORMATION:/label= Pep30-2
 /note= "Pep30-2 is a synthetically prepared truncated
 form of a TNF-alpha analog comprising the human T cell
 epitope P30 and flanking portions of human TNF-alpha"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

Ala	Leu	Leu	Ala	Asn	Phe	Asn	Asn	Phe	Thr	Val	Ser	Phe	Trp	Leu	Arg
1				5					10					15	
Val	Pro	Lys	Val	Ser	Ala	Ser	His	Leu	Glu	Gln	Val	Leu	Phe	Lys	
			20					25					30		

(2) INFORMATION FOR SEQ ID NO: 40:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

(ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION:1..31
- (D) OTHER INFORMATION:/label= Pep30-3
/note= "Pep30-3 is a synthetically prepared truncated form of a TNF-alpha analog comprising the human T cell epitope P30 and flanking portions of human TNF-alpha"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

Tyr	Ser	Gln	Val	Leu	Phe	Asn	Asn	Phe	Thr	Val	Ser	Phe	Trp	Leu	Arg
1				5					10					15	
Val	Pro	Lys	Val	Ser	Ala	Ser	His	Leu	Glu	Val	Ser	Tyr	Gln	Thr	
			20					25					30		

(2) INFORMATION FOR SEQ ID NO: 41:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

(ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION:1..31

(D) OTHER INFORMATION:/label= Pep30-4

/note= "Pep30-4 is a synthetically prepared truncated form of a TNF-alpha analog comprising the human T cell epitope P30 and flanking portions of human TNF-alpha"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41:

Gln	Arg	Glu	Thr	Pro	Phe	Asn	Asn	Phe	Thr	Val	Ser	Phe	Trp	Leu	Arg
1				5					10					15	
Val	Pro	Lys	Val	Ser	Ala	Ser	His	Leu	Glu	Lys	Gly	Asp	Arg	Leu	
			20					25					30		

(2) INFORMATION FOR SEQ ID NO: 42:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION:1..31
- (D) OTHER INFORMATION:/label= Pep30-5
- /note= "Pep30-5 is a synthetically prepared truncated form of a TNF-alpha analog comprising the human T cell epitope P30 and flanking portions of human TNF-alpha"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42:

Glu	Lys	Gly	Asp	Arg	Phe	Asn	Asn	Phe	Thr	Val	Ser	Phe	Trp	Leu	Arg
1				5					10					15	
Val	Pro	Lys	Val	Ser	Ala	Ser	His	Leu	Glu	Gly	Ile	Ile	Ala	Leu	
			20					25					30		

P A T E N T C L A I M S

1. A modified human TNF α molecule capable of raising neutralizing antibodies towards unmodified human TNF α following administration of said modified TNF α to a human host, wherein one or more peptide fragments of the human TNF α molecule has been substituted by one or more peptides known to contain immunodominant T cell epitopes or a truncated form of said molecule containing the immunodominant epitope and one or both flanking regions of the human TNF α -molecule comprising at least one TNF α B cell epitope, wherein the substitution has been made in regions of the TNF α molecule which do not comprise the B and G strands of the back β -sheet of the molecule.
2. Modified human TNF α molecule according to claim 1, wherein the substitution has been made in regions of the TNF α molecule which does not comprise any of the strands of the back β -sheet.
3. Modified human TNF α molecule according to claim 1, wherein the substitution has been made in regions of the TNF α molecule which involve a segment of the D strand of the back β -sheet.
4. Modified human TNF α molecule according to claim 2, wherein the substitution comprises at least a segment of the H strand of the front β -sheet and of the connecting loop to the I strand, preferably amino acids 132 to 146.
5. Modified human TNF α molecule according to claim 1, wherein the substitution comprises segments of the H and I strands and the entire connecting loop, preferably amino acids 132 to 152
6. Modified human TNF α molecule according to claim 1, wherein the substitution comprises a segment of the D

strand, at least a segment of the E strand and the entire connecting loop, preferably amino acids 65 to 79 or 64 to 84.

5 7. Modified human TNF α molecule according to claim 1, wherein the substitution comprises the entire C' and C strands and a segment of the D strand, preferably amino acids 40 to 60.

10 8. Modified human TNF α molecule according to claim 2, wherein the substitution comprises at least a segment of the E strand and of the front β -sheet of one or both of the connecting loops, preferably amino acids 76 to 90.

9. Modified TNF α according to claim 2 or 4, having the amino acid sequence shown in SEQ ID NO:8.

15 10. Modified TNF α according to claim 2 or 8, having the amino acid sequence shown in SEQ ID NO:10.

11. Modified TNF α molecule according to claim 3 or 6, having the amino acid sequence shown in SEQ ID NO:4 or SEQ ID NO:16.

20 12. Modified TNF α according to claim 5, having the amino acid sequence shown in SEQ ID NO:20.

13. Modified TNF α according to claim 3 or 7, having the amino acid sequence shown in SEQ ID NO:14.

25 14. Modified human TNF α molecule according to any of the preceding claims, wherein the inserted T cell epitope is promiscuous and known to be immunogenic in a majority of human HLA class II types.

15. Modified human TNF α molecule according to claim 14, wherein the epitope is derived from Tetanus toxoid, preferably epitope P2 and/or P30.

16. Dimers, oligomers or multimers of the modified human TNF α molecule according to any one of claims 1-13.
17. An isolated DNA molecule that codes for a modified TNF α molecule according to any one of claims 1 to 13.
- 5 18. A vector which comprises the isolated DNA molecule according to claim 17.
19. An expression vector which comprises the isolated DNA molecule according to claim 17 operatively linked to an expression control sequence.
- 10 20. A host, which is transformed with the expression vector of claim 19.
21. A host according to claim 20 which is selected from strains of bacteria, yeast, or other fungi and insect, mammalian or avian cell lines.
- 15 22. A method of producing a modified human TNF α molecule according to any one of claims 1-10, which comprises growing the host cells of claim 20 under suitable conditions permitting production of the modified TNF α and recovering the modified TNF α so produced.
- 20 23. A modified human TNF α molecule according to any of claims 1 - 13 in the form of a fusion protein with an adjuvant molecule, preferably an immunologically active adjuvant, such as GM-CSF, HSP70 or interleukin.
- 25 24. A vaccine against TNF α , comprising an immunogenic amount of one or more modified human TNF α molecules according to any of claims 1 - 13 and optionally a pharmaceutically acceptable adjuvant, such as Adju-Phos, aluminium hydroxide, calcium phosphate, muramyl dipeptide or iscom.

25. A vaccine according to claim 24 for the prevention or treatment of TNF α -dependent diseases such as chronic inflammatory diseases, such as rheumatoid arthritis and inflammatory bowel diseases, including Crohn's disease, and cancer, disseminated sclerosis, diabetes, psoriasis, osteoporosis and asthma.

26. A vaccine against TNF α comprising isolated DNA which codes for the modified human TNF α molecule according to any one of claims 1 - 13 inserted in a suitable expression vector.

27. A vaccine according to claim 26 containing a construct comprising a non-infectious non-integrating DNA sequence encoding a modified TNF α molecule according to any of claims 1 - 13 operatively linked to a promoter sequence which can control the expression of said DNA sequence in humans, in an amount sufficient that uptake of said construct occurs, and sufficient expression occurs to induce a neutralizing antibody response against TNF α .

28. A vaccine according to claim 26, comprising a viral expression vector, such as a retroviral expression vector.

29. A vaccine according to any one of claims 24 - 28 for oral or parenteral, e.g. subcutaneous, intramuscular or intradermal administration.

30. The use of antibodies raised by administering a vaccine according to any one of claims 24-29, preferably monoclonal antibodies.

31. Diagnostic use of antibodies according to claim 30.

32. A method of testing human body fluids for the presence of TNF α which comprises contacting a composition containing modified TNF α according to any one of claims

1-13 with a sample of human body fluid and determining whether said antibodies bind to TNF α in said sample.

33. A diagnostic method for TNF α -related diseases employing an in vitro immunoassay to detect TNF α in human
5 body fluids.

34. The method of claim 32 or 33 which comprises the use of a sandwich assay, ELISA assay or equivalent assay, which can be unamplified or amplified, e.g. using avidin/biotin technology.

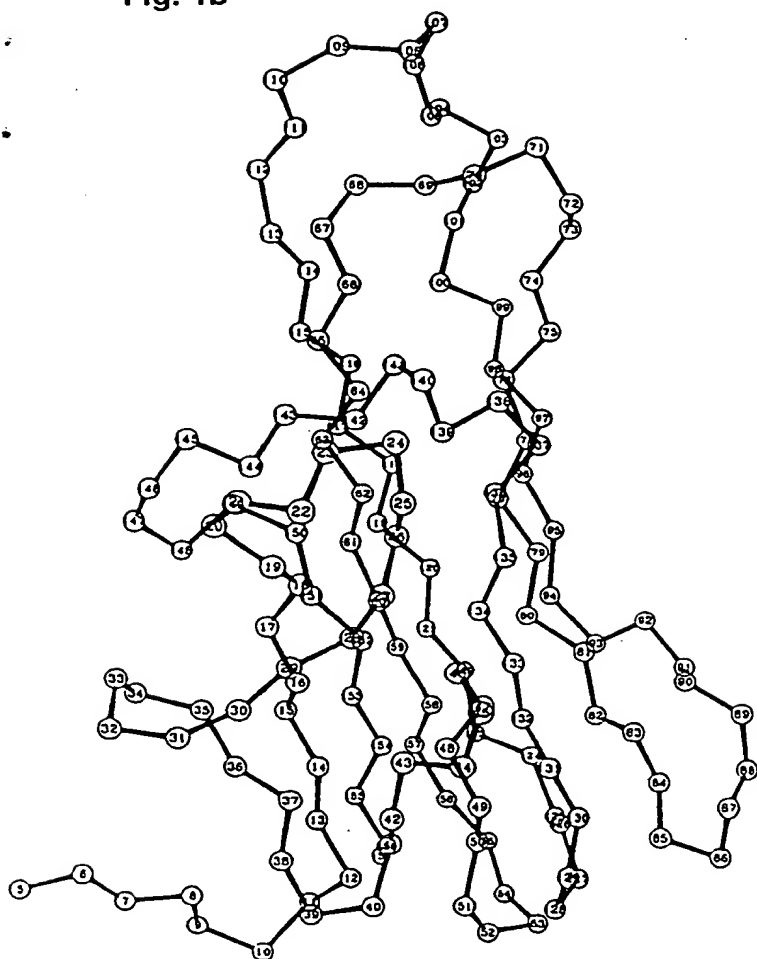
A B S T R A C T

A modified human TNF α molecule capable of raising neutralizing antibodies towards unmodified human TNF α following administration to a human host, wherein one or
5 more peptide fragments of the TNF α molecule has been substituted by a peptide containing immunodominant T cell epitopes, the substitution having been made in regions of the TNF α molecule not comprising the B and G strands of the back β -sheet and preferably not any of the strands of
10 the back β -sheet.

The modified human TNF α molecules or DNA encoding them may be formulated as vaccines against TNF α optionally with pharmaceutically acceptable adjuvants, for the prevention or treatment of chronic inflammatory diseases,
15 such as rheumatoid arthritis and inflammatory bowel diseases, cancer, disseminated sclerosis, diabetes, psoriasis, osteoporosis or asthma.

Human body fluids may be tested for the presence of TNF α by contact with a composition containing the modified
20 TNF α .

Fig. 1b



Crystal Structure of TNF

Fig. 1a

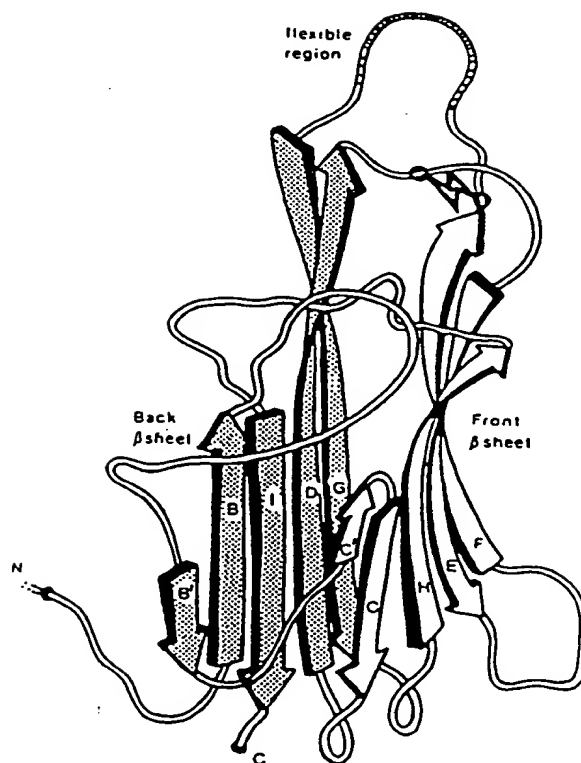


Fig. 1c

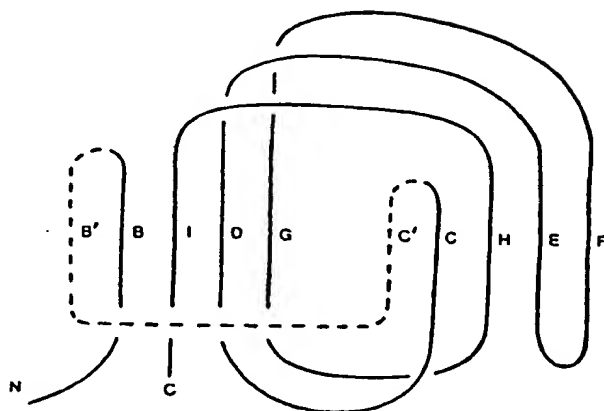


Fig. 2

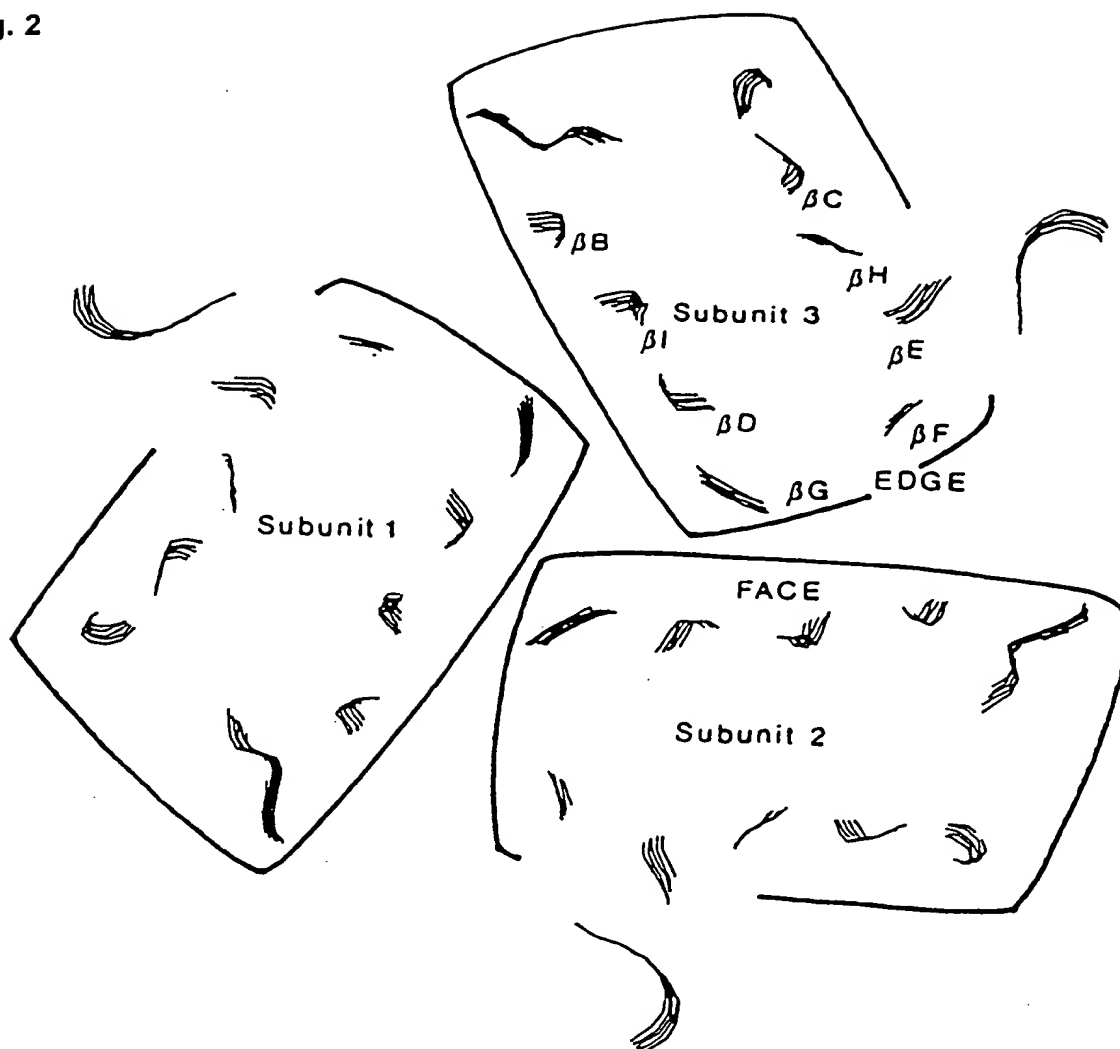


Fig. 3b

Amino acid sequence for human TNF α 8

Accession code: Swissprot P01375

```
-76  MSTESMIRDV ELAEEALPKK TGGPQGSRRCL FLSLESELI VAGATTLECL
-26  LHEGVIGPQR EEFPRDLSLI SPLAQA
. 1  VRSSSRTPSD KPAHVVANP QAEGQLQWLN RRANALLANG VELRDNQLVV
51  PSEGLYLIYS QVLFKGQGCP STHVLLTHTI SRIAVSYQTK VNLLSAIKSP
101 CQRETPEGAE AKPWEPIYL GGVFQLEKGD RLSAEINRPD YLDFAESGQV
151 YFGIIAL
```

Conflicting sequence F→S at position -14. Disulphide bond between Cys69-101. Signal anchor sequence -41 to -21 (underlined). Myristylation on Lys-58/-57.

Fig. 3a

1	cacaccctga	caagctgcca	ggcaggttct	cttctctctca	catactgacc
51	cacggctcca	ccctctctcc	cctggaaagg	acaccatgag	cactgaaage
101	atgatccggg	acgtggagct	ggccgaggag	gcgctcccca	agaagacagg
151	ggggccccag	ggctccaggc	ggtgcttggt	cctcagcctc	ttctccttcc
201	tgatcgtggc	aggcgccacc	acgtctttct	gcctgctgca	ctttggagtg
251	atcggccccc	agaggggaaga	gtcccccagg	gacctctctc	taatcagccc
301	tctggcccag	gcagtcagat	catcttctcg	aaccccgagt	gacaagcctg
351	tagcccatgt	tgtagcaaac	cctcaagctg	aggggcagct	ccagtggctg
401	aaccgcctgg	ccaatgccct	cctggccaat	ggcgtggagc	tgagagataa
451	ccagctgggtg	gtgccatcag	agggcctgta	cctcatctac	tcccagggtcc
501	tcttcaaggg	ccaaggetgc	ccctccaccc	atgtgctcct	caccacacacc
551	atcagccgca	tcgcctctc	ctaccagacc	aagggtcaacc	tcctctctgc
601	catcaagagc	ccctgccaga	gggagacccc	agaggggget	gaggccaagc
651	cctgggtatga	gcccattctat	ctgggagggg	tcttccagct	ggagaaggggt
701	gaccgactca	gcgctgagat	caatcgcccc	gactatctcg	actttgcccga
751	gtctgggcag	gtctactttg	ggatcattgc	cctgtgagga	ggacgaacat
801	ccaaccttcc	caaacgcctc	ccctgcccc	atccctttat	tacccctcc
851	ttcagacacc	ctcaacctct	tctggctcaa	aaagagaatt	gggggcttag
901	ggtcggaacc	caagcttaga	actttaagca	acaagaccac	cacttcgaaa
951	cctgggattc	aggaatgtgt	ggcctgcaca	gtgaagtgtc	ggcaaccact
1001	aagaattcaa	actggggcct	ccagaactca	ctggggccta	cagctttgat
1051	ccctgacatc	tggaatctgg	agaccaggga	gcctttgggt	ctggccagaa
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1151	taggccttcc	tctctccaga	tgtttccaga	cttctctgag	acacggagcc
1201	cagccctccc	catggagcca	gtccctctca	tttatgtttg	cacttgtgat
1251	tattttattat	ttattttatta	tttattttatt	tacagatgaa	tgtattttatt
1301	tgggagaccg	gggtatcctg	ggggacccaa	tgtaggagct	gccttggtc
1351	agacatgttt	tccgtgaaaa	cggaggctga	acaataggct	gttccccatgt
1401	agcccccctgg	cctctgtgcc	ttcttttgat	tatgtttttt	aaaatattat
1451	ctgatttaagt	tgtctaaaca	atgctgattt	ggtgaccaac	tgtcactcat
1501	tgctgaggcc	tctgctcccc	agggagttgt	gtctgtaatc	ggcctactat
1551	tcagtggcga	gaaataaagg	ttgcttagga	aagaa	

Fig. 4a

Location of inserted epitopes



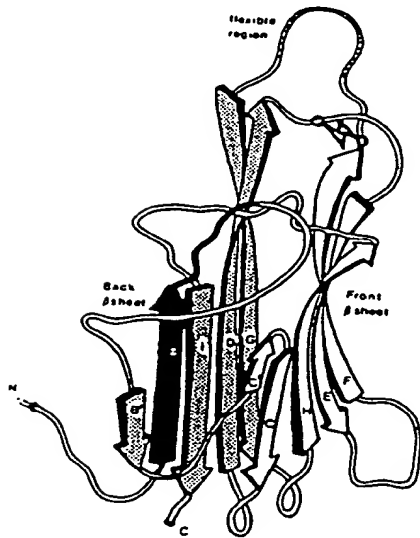
Fig. 4b

#	1	11	21	31	41	51	61	71
WT	VRSSRTPSD	KPAHVVANP	QAEGLQWLN	RRANALLANG	VELRDNQLV	PSEGLYLIYS	QVLFKGQGCP	STHVLLTHTI
2-1	-----Q	YIKANSKFIG	ITEL	-----	-----	-----	-----	-----
2-3	-----	-----	-----	-----	-----	-----	-----QYIKANSKFIGITEL	-----
2-4	-----	-----	-----	-----	-----	-----	-----	-----
2-5	-----	-----	-----	-----	-----	-----	-----	-----
2-7	-----	-----	-----	-----	-----	-----	-----	-----QYIKA
30-1	-----F	NNFTVSFWLR	VPKVSASHLE	-----	-----	-----	-----	-----
30-2	-----	-----	-----F	NNFTVSFWLR	VPKVSASHLE	-----	-----	-----
30-3	-----	-----	-----	-----	-----	-----FNNFTVS	FWLRVPKVS	A
30-4	-----	-----	-----	-----	-----	-----	-----	-----
30-5	-----	-----	-----	-----	-----	-----	-----	-----

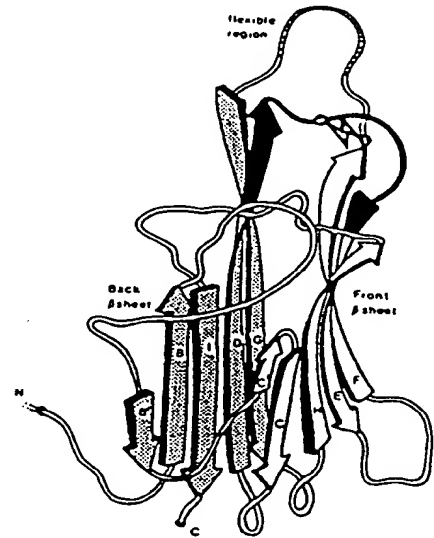
#	81	91	101	111	121	131	141	151
WT	SRIAVSYQTK	VNLLSAIKSP	CQRETPEGAE	AKPWEPIYL	GGVFQLEKGD	RLSAEINRPD	YLDFAESGQV	YFGIIAL
2-1	-----	-----	-----	-----	-----	-----	-----	-----
2-3	-----	-----	-----	-----	-----	-----	-----	-----
2-4	-----	-----	-----	-----QYIKANS	KFIGITEL	-----	-----	-----
2-5	-----	-----	-----	-----	-----	-----QYIKANSKF	IGITEL	-----
2-7	NSKFIGITEL	-----	-----	-----	-----	-----	-----	-----
30-1	-----	-----	-----	-----	-----	-----	-----	-----
30-2	-----	-----	-----	-----	-----	-----	-----	-----
30-3	SHLE	-----	-----	-----	-----	-----	-----	-----
30-4	-----	-----	-----FNNFT	VSFWLRVPKV	SASHLE	-----	-----	-----
30-5	-----	-----	-----	-----	-----	-----FNNFTVSFW	LRVPKVSASH	LE

Fig. 5a

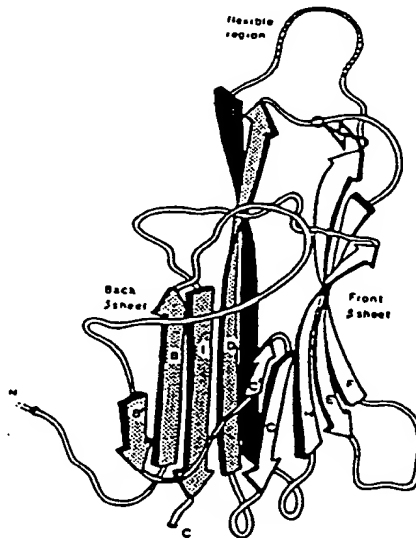
TNF α analogs with the P2 epitope inserted.



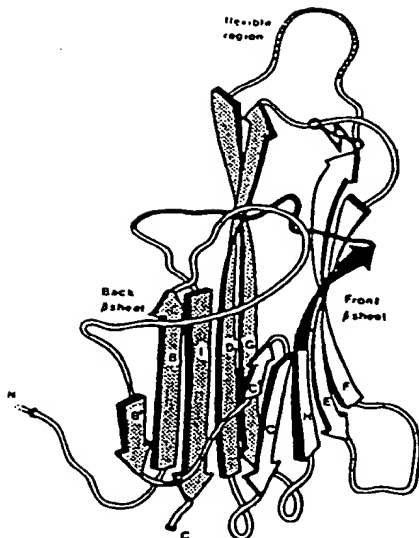
TNF2-1



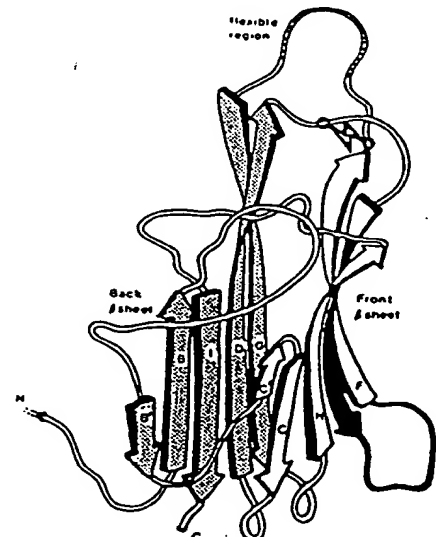
TNF2-3



TNF2-4



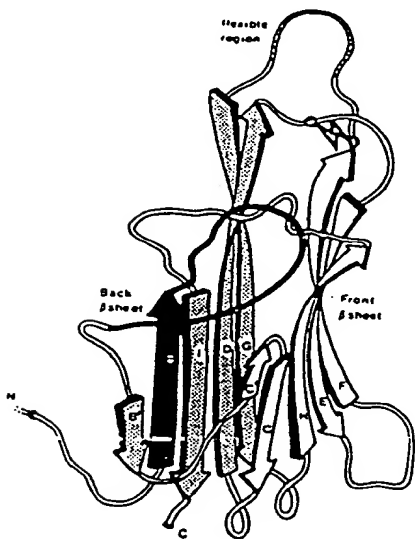
TNF2-5



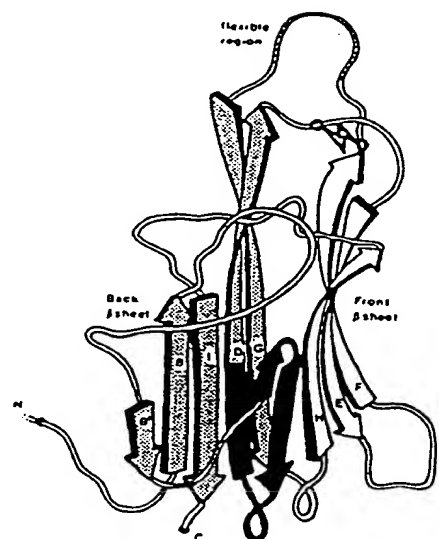
TNF2-7

Fig. 5b

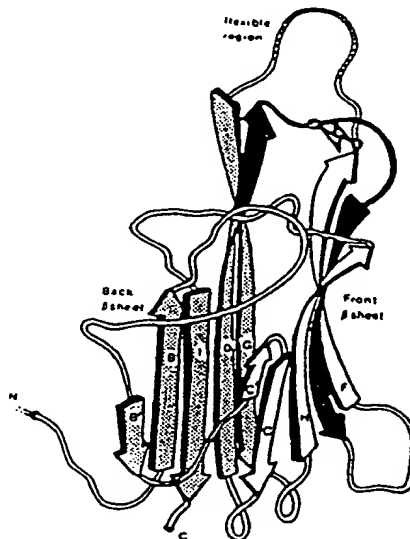
TNF α analogs with the P30 epitope inserted.



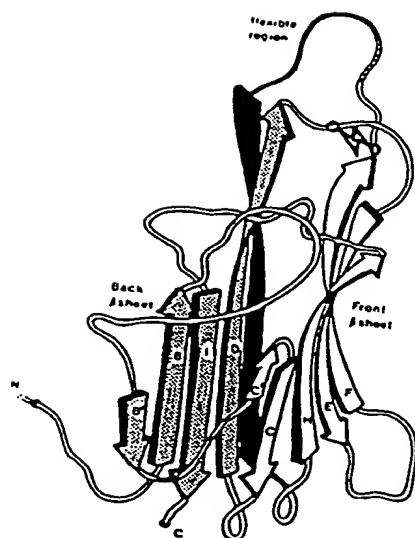
TNF30-1



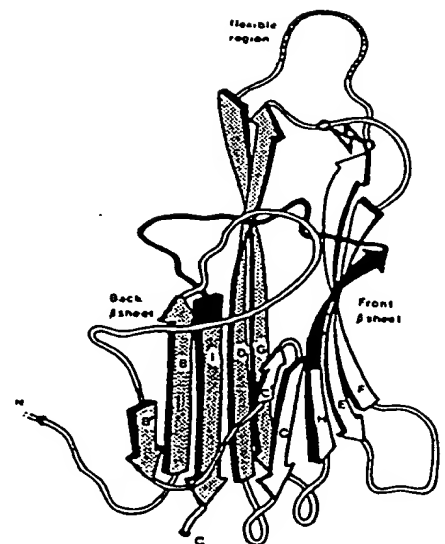
TNF30-2



TNF30-3



TNF30-4



TNF30-5

٢

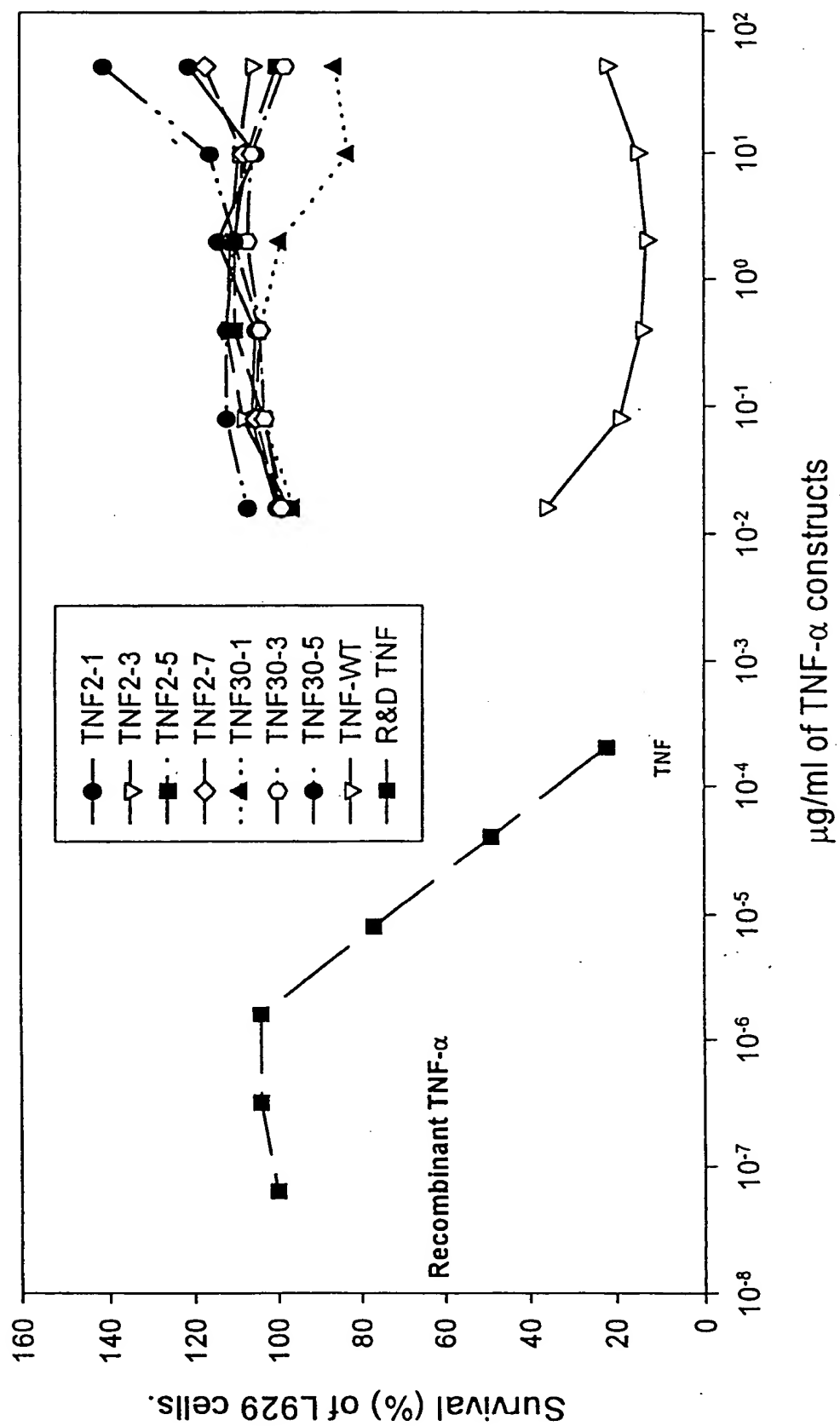


Fig. 7

The anti human TNF α antibody response in rabbits.

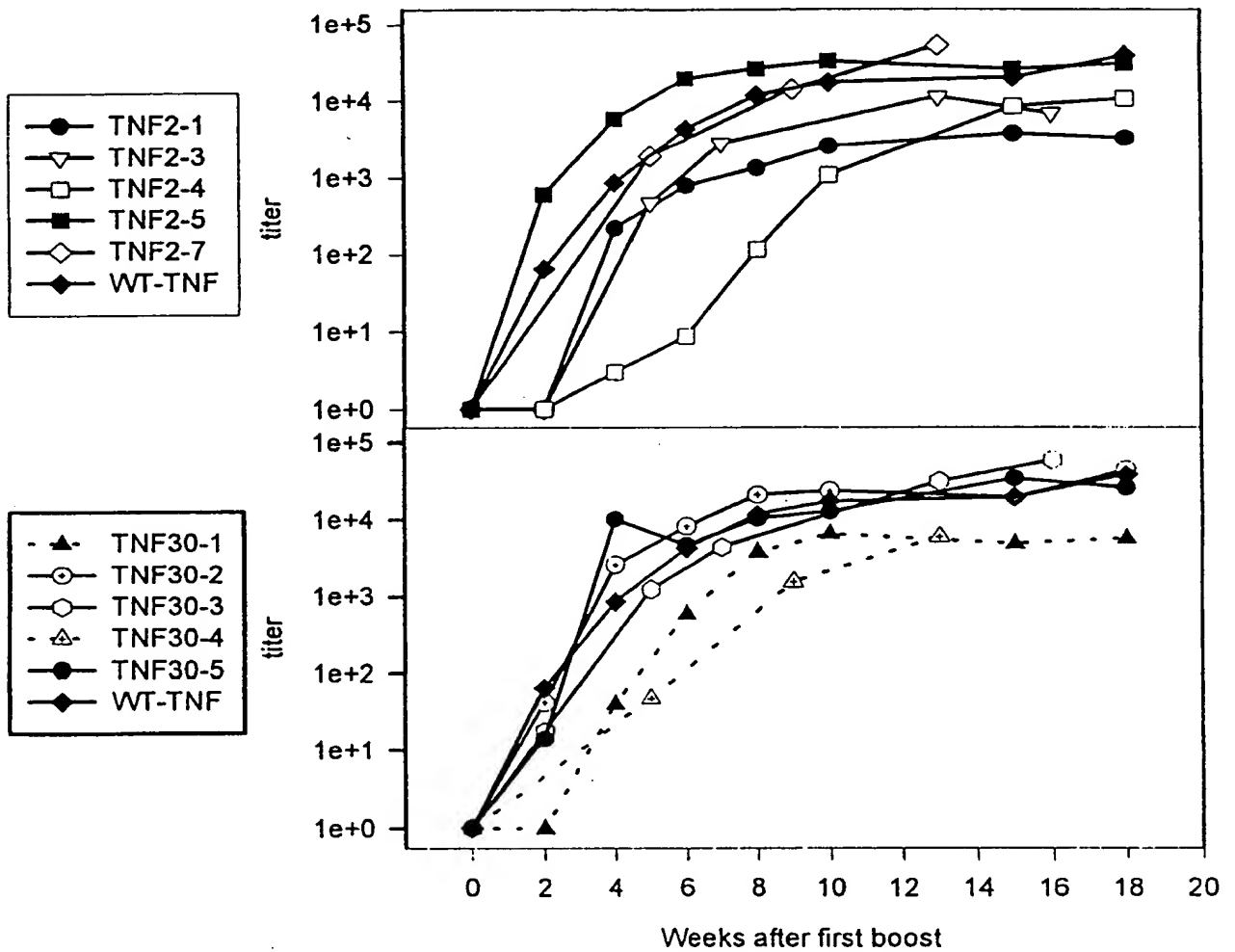


Fig. 8

The ability of P2/P30 modified human TNF α molecules to induce neutralizing antibodies as measured in the L929 cell assay.

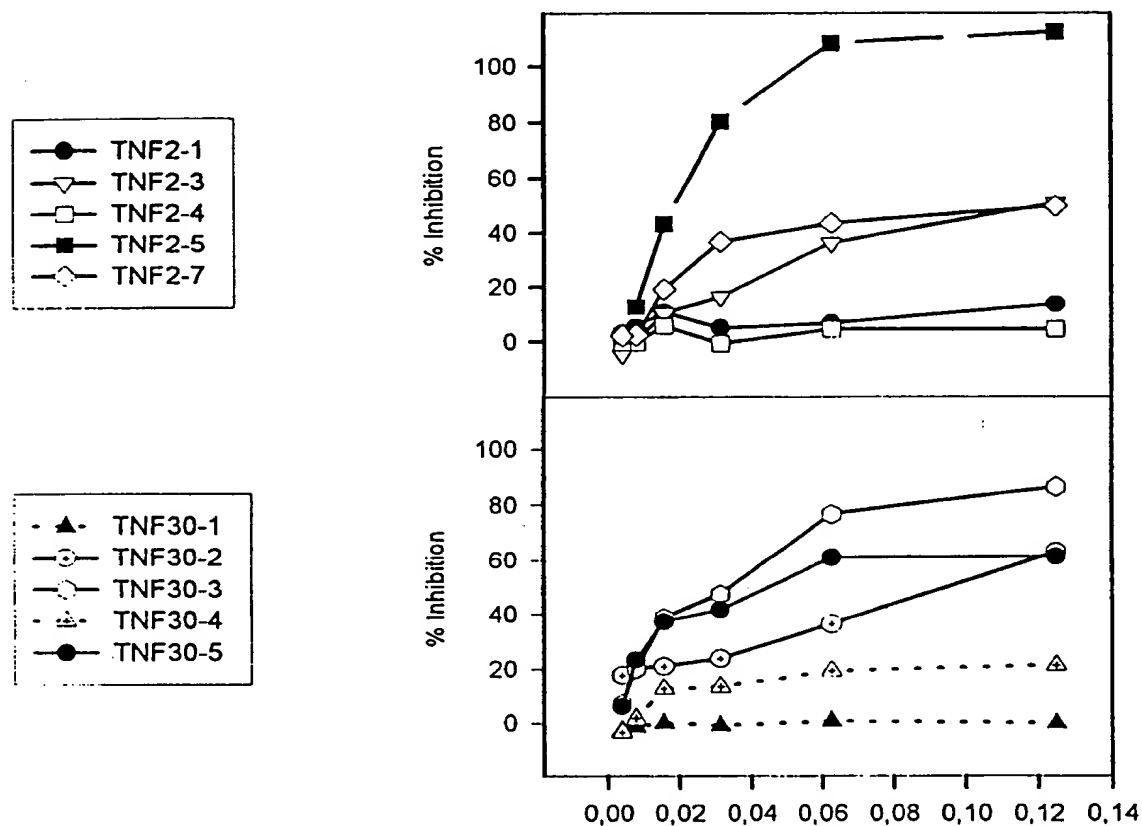


Fig. 9

The ability of P2/P30 modified human TNF α molecules to induce neutralizing antibodies as measured in the receptor assay

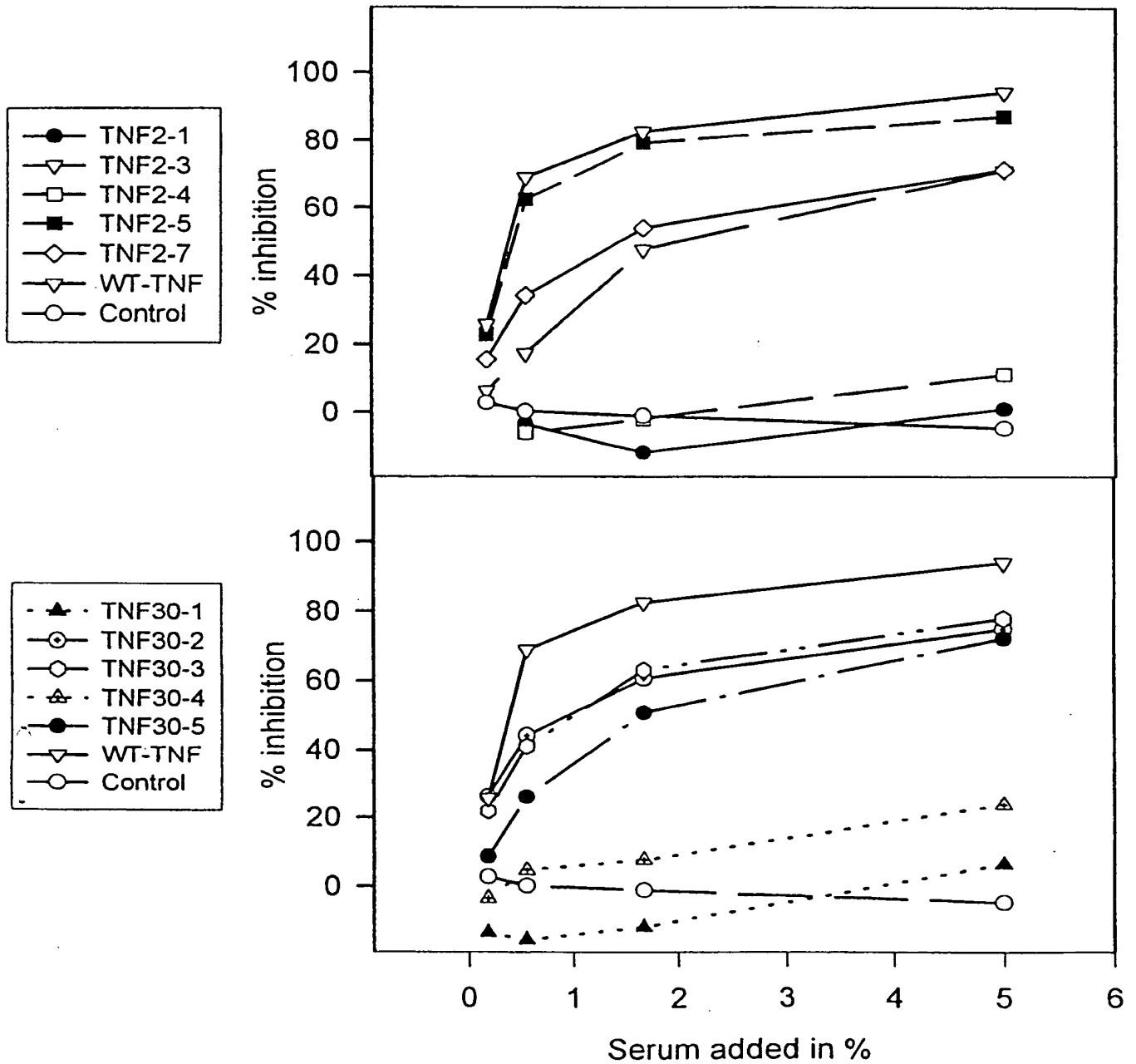


Fig. 10

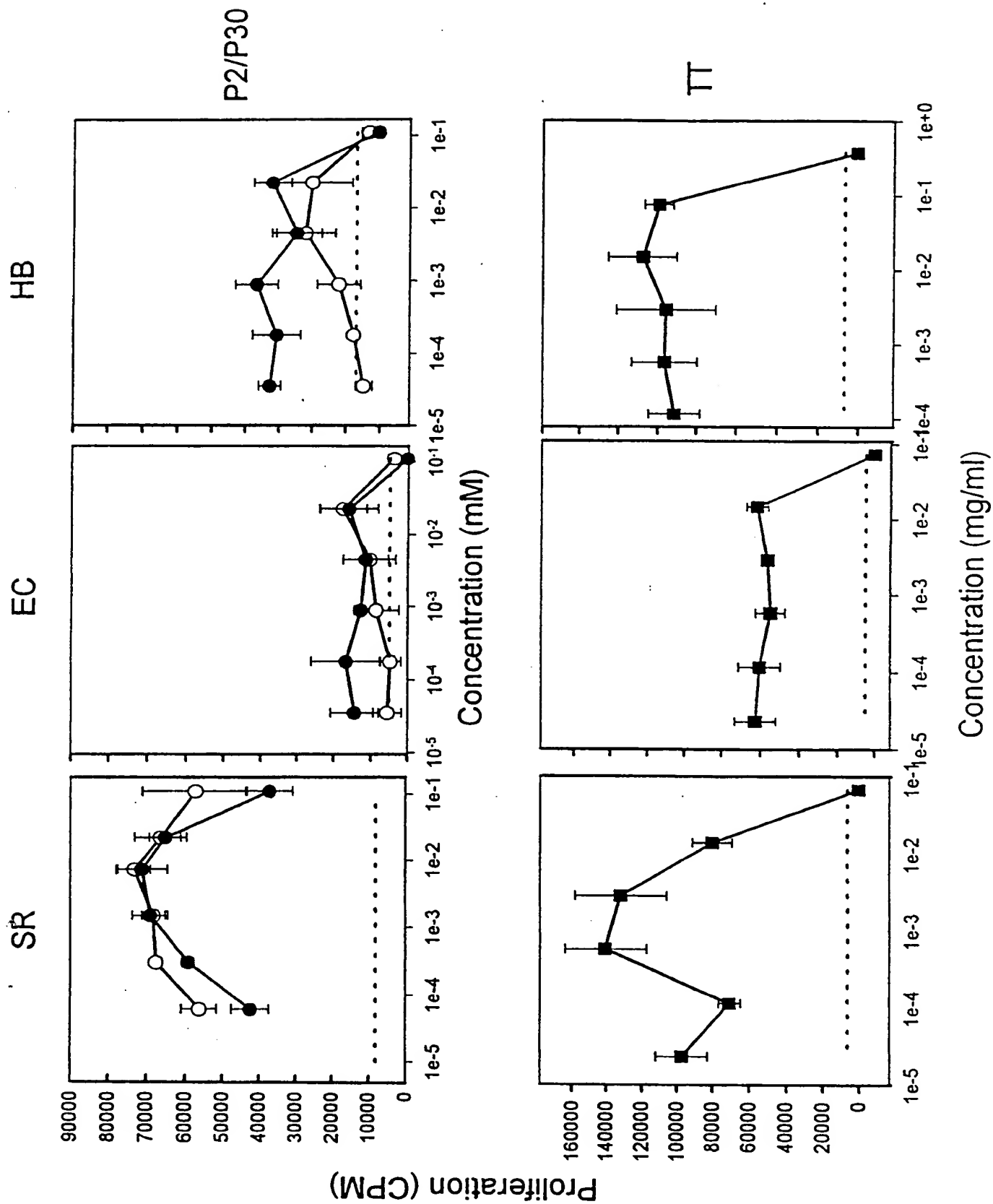


Fig. 11

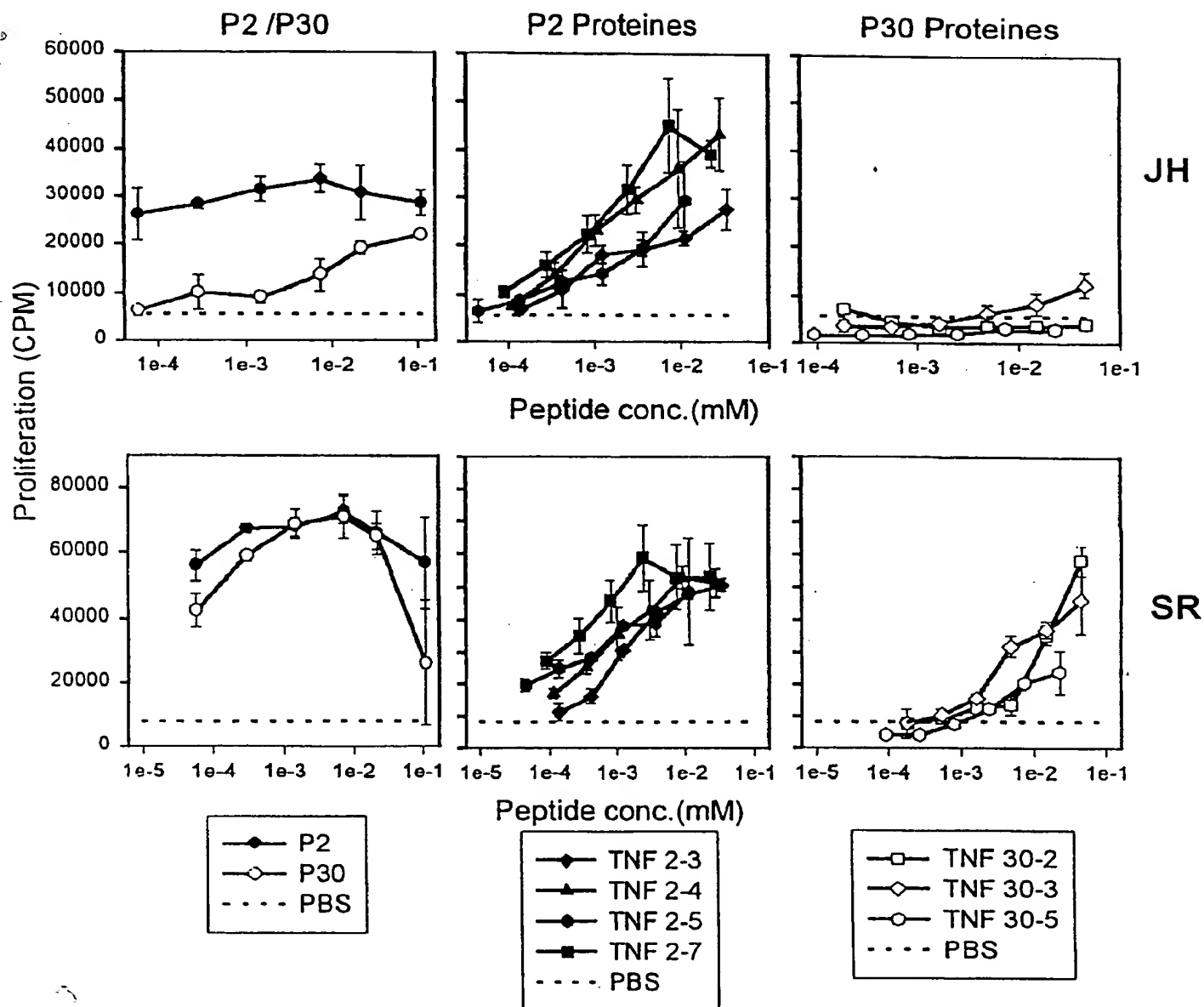


Fig. 12

PBMC Assays

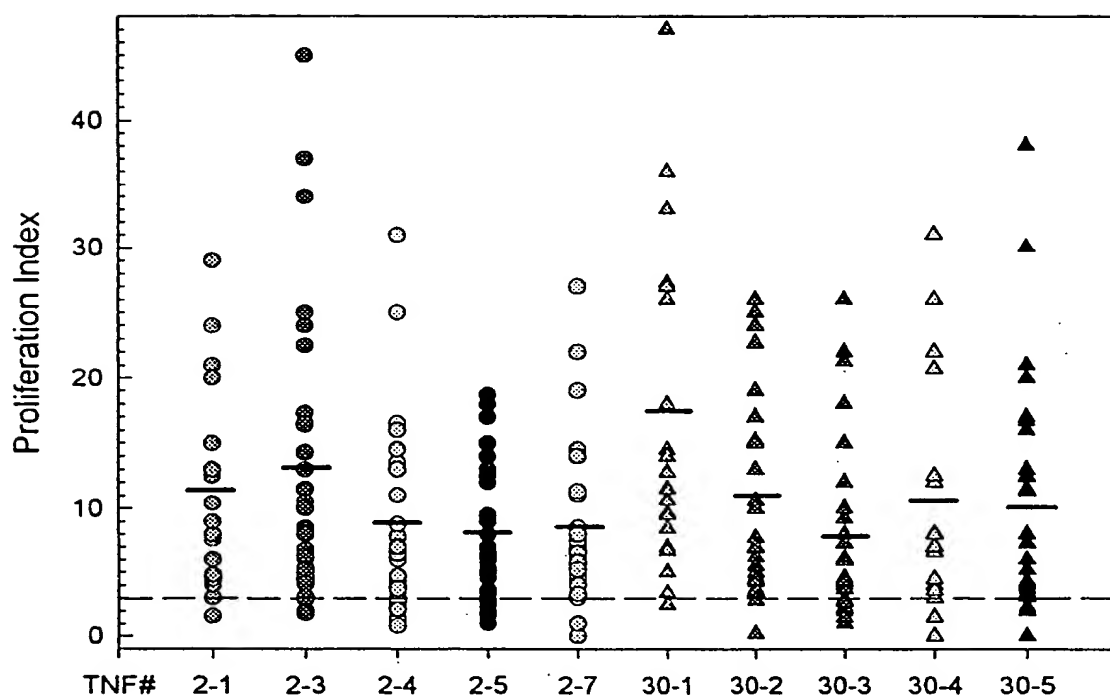


Fig. 13

The PBMC response against P2 and P30 modified TNF α proteins in P2 and P30 specific responders, respectively.

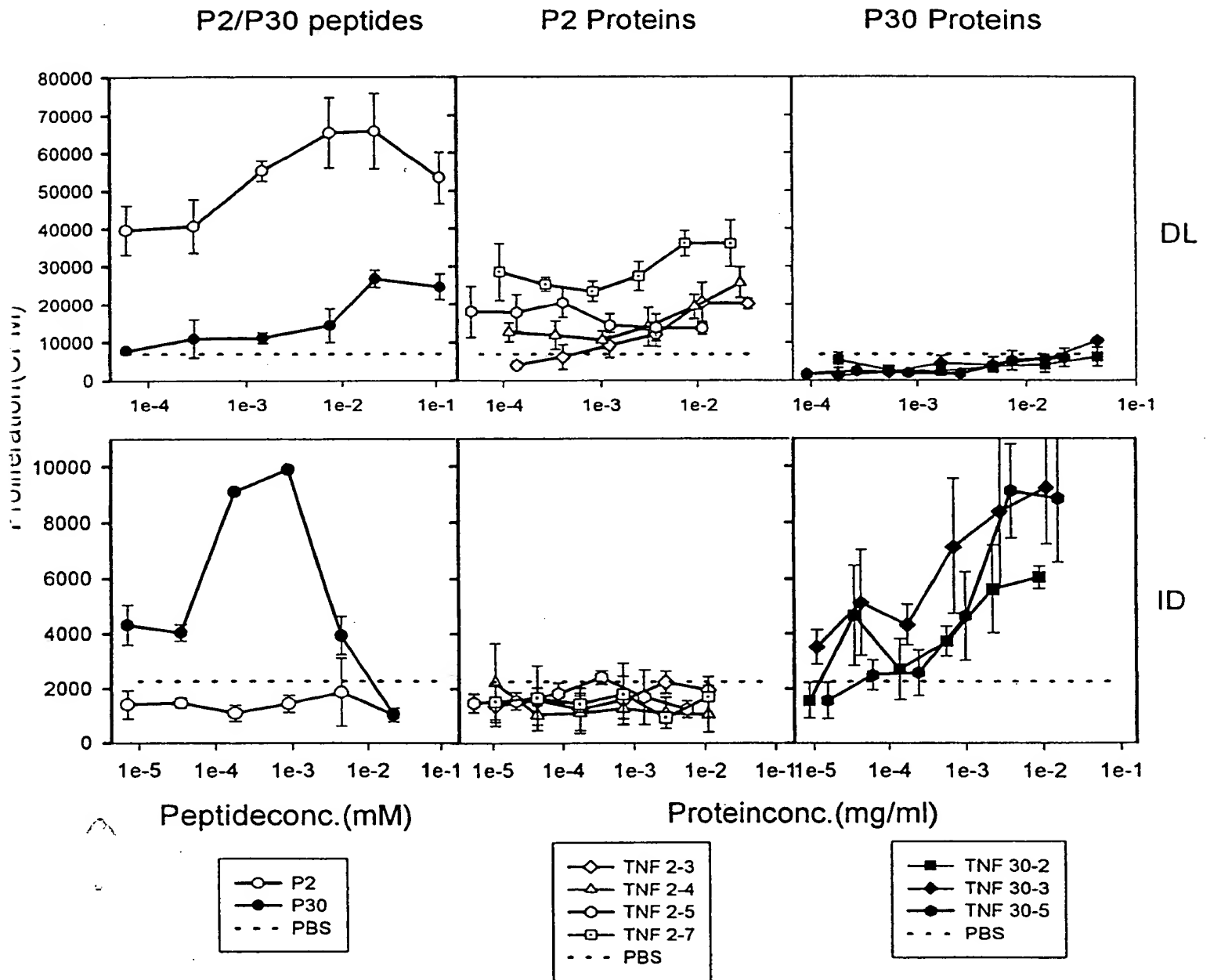


Fig. 14

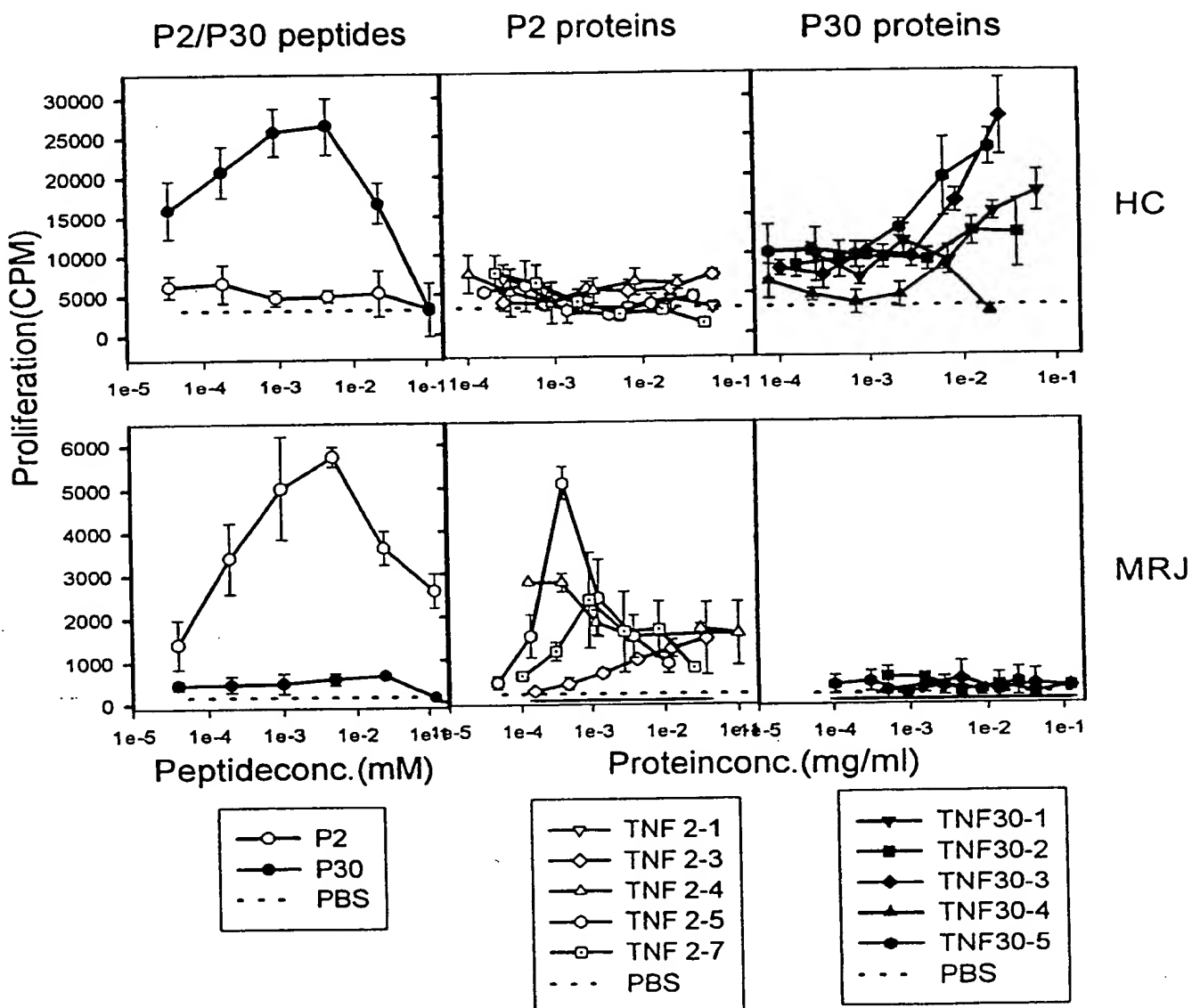
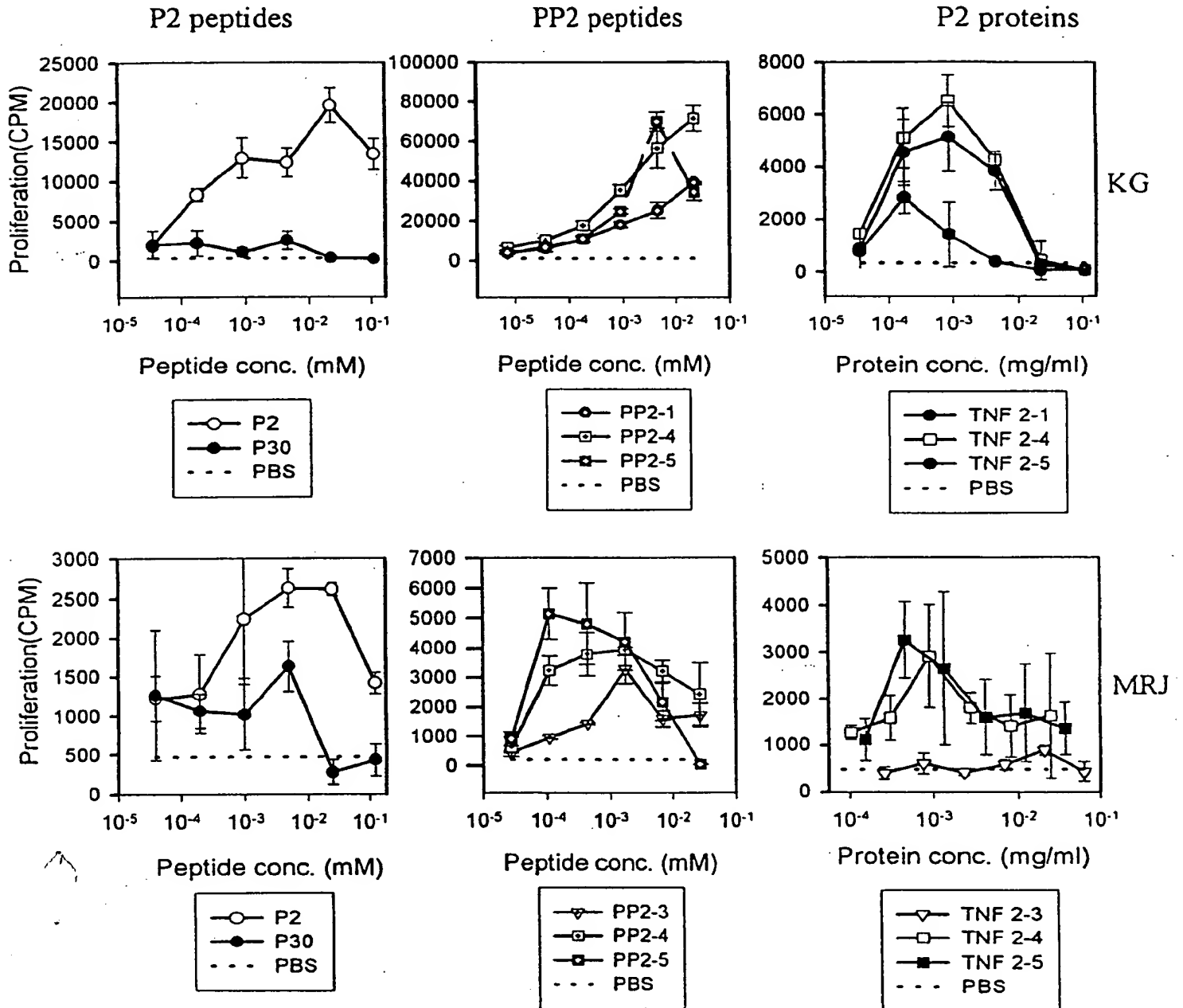
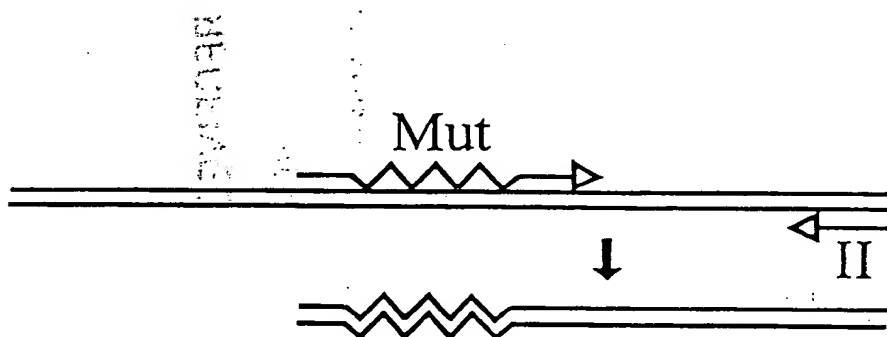
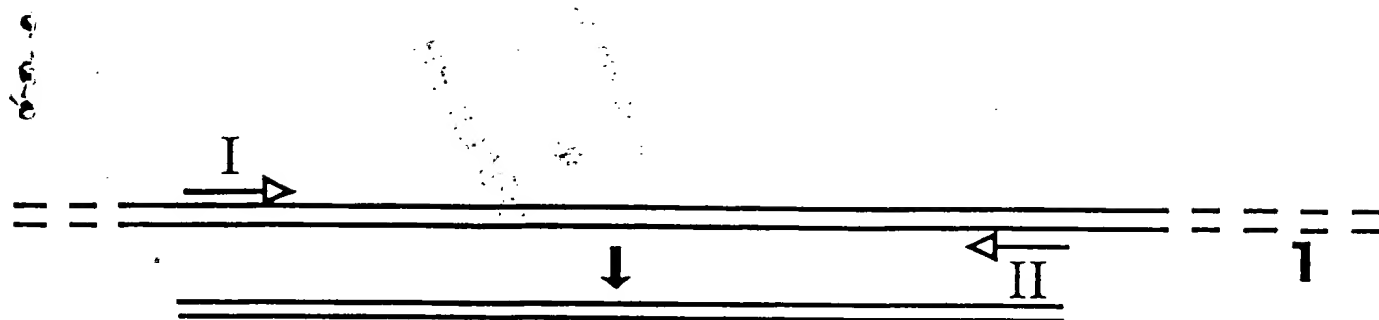


Fig. 15

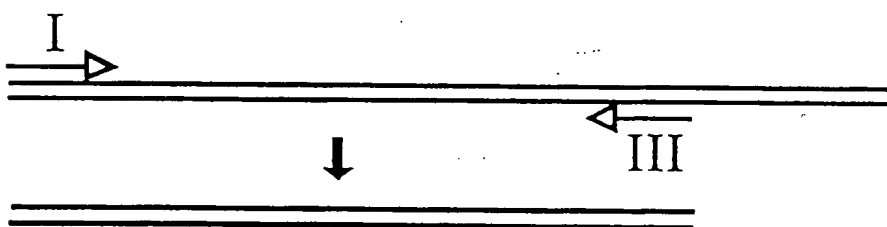
The influence of flanking amino acids on the T cell recognition of P2 and P30



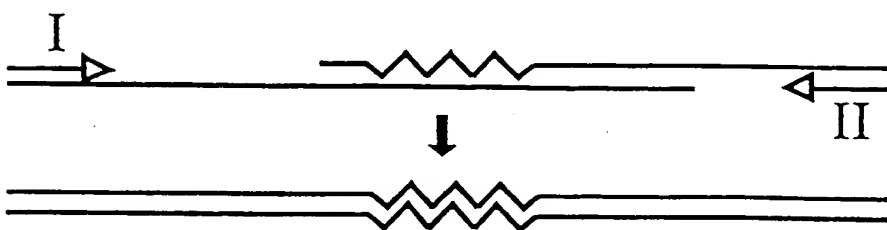
Mutation Strategy



2a



2b



3